Toll-like Receptor Signalling in Rheumatoid Arthritis

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Summary

Toll-like receptors (TLRs) have been identified as key recognition structures of the innate immune system. The activation of TLRs by infectious organisms or endogenous ligands induces the production of proinflammatory cytokines and upregulates costimulatory molecules of antigen presenting cells. Considering the important role of TLR signalling in linking innate and adaptive immunity, it has been proposed, that dysregulated TLR signalling might be associated with autoimmune disease such as rheumatoid arthritis (RA). RA is a chronic systemic inflammatory disease leading to irreversible joint destruction and loss of function. Synovial fibroblasts (RASF), which are the major cellular subset of the hyperplastic lining in rheumatoid joints, play a key role in the pathogenesis of RA by releasing large quantities of matrix degrading enzymes, proinflammatory cytokines and chemokines. Since TLR ligands have been shown to be powerful inducers of such proinflammatory and destructive mediators we hypothesized that the activated and invasive phenotype characteristically seen in RASF might be a consequence of active TLR signalling.

In this work, it is demonstrated that RASF express functional TLR1, 2, 3, 4, 5 and 6. TLR3 was the most abundant TLR expressed in RASF and activation of TLR3 led to the most marked induction of interleukin (IL) -6, CCL5, CXCL10, matrix metalloproteinase (MMP) 3, MMP13 as well as TLR3 itself. TLR3 was more abundant in RA than in OA synovial tissues and was predominantly expressed by RASF in the synovial lining layer. dsRNA released from necrotic synovial fluid cells was found to act as endogenous TLR3 ligand for RASF. These results suggest that endogenous dsRNA might be responsible for sustained TLR3 activation in joints of patients with RA.

Activation of TLR3 signalling in RASF led to the upregulation of the recently described pre-B-cell colony enhancing factor (PBEF). PBEF is a multifunctional protein having nicotinamide phosphoribosyltransferase, adipokine and cytokine activities. Levels of PBEF in synovial fluids and serum were found to be elevated in RA compared to OA patients and correlated significantly with the degree of inflammation. PBEF was found to act by itself as proinflammatory mediator by triggering the release of cytokines, chemokines and destructive enzymes in RASF and primary monocytes. Furthermore, PBEF knockdown in RASF significantly inhibited basal and TLR ligand induced production of IL-6, IL-8, MMP1 and MMP3. These results suggest that PBEF plays a key role as a mediator of innate immune

pathways in chronic synovial inflammation and joint destruction and identify PBEF as a possible therapeutic target for the treatment of RA.

TLR3 as well as TLR2 and 4 ligands also induced the expression of p19, a subunit of IL-23, in cultured RASF. The cytokine IL-23, composed of a p19 and p40 subunit, is suggested to be involved in the pathogenesis of RA, dependent on the promotion and proliferation of IL-17 producing Th17 T-cells. The IL-23 p19 subunit was expressed in RA synovial tissues predominantly at sites of invasion and in the synovial lining layer. However, no heterodimeric IL-23 was found at these sites and RASF did not express p40 following TLR activation. Correspondingly, soluble IL-23 was found at very low levels in synovial fluids and sera of patients with RA. The differential expression of p19 and p40 suggests that p19 does not necessarily indicate the presence of IL-23, as it has been proposed in earlier reports, and leaves open the possibility of a p40 independent involvement of p19 in the pathogenesis of RA.

In summary, there is evidence for an implication of TLR3 and TLR3 dependent gene regulation pathways in the pathogenesis of RA. Therefore, targeting TLR3, TLR signalling molecules and especially cytokines downstream of TLR activation, such as PBEF, seems to be a promising approach to treat arthritis.

Zusammenfassung

Die Toll-like Rezeptoren (TLR) sind Strukturen des angeborenen Immunsystems. Die Aktivierung von TLR durch pathogenen Mikroorganismen und wirtseigene Moleküle, löst die Produktion von proinflammatorischen Zytokinen und die Aufregulierung von co-stimulatorischen Molekülen auf. Dabei steuern die TLR neben der angeborenen auch die erworbene Immunantwort und es wird vermutet, dass eine fehl regulierte TLR Signalkaskade mit Autoimmunerkrankungen assoziiert ist. Bei der rheumatoiden Arthritis (RA) handelt es sich um eine chronisch-entzündliche, systemische Autoimmunerkrankung, charakterisiert durch unkontrollierte Proliferation von Synovialgewebe mit daraus resultierender Gelenkdestruktion. Die synovialen Fibroblasten bei Patienten mit RA (RASF) sollten als Schlüsselzellen für die Gelenkdestruktion betrachtet werden, da diese direkt durch Sekretion matrixdegradierender Enzyme und durch Produktion von proinflammatorischen Zytokinen und Chemokinen dazu beitragen. Der aktivierte und invasive Phänotyp von synovialen Fibroblasten in RA Patienten könnte die Konsequenz einer persistierenden TLR Aktivierung sein.

In der vorliegenden Arbeit wird gezeigt, dass RASF funktionsfähige TLR1, 2, 3, 4, 5 und 6 besitzen. Dabei war TLR3 am höchsten exprimiert und die Aktivierung von RASF via TLR3 führte zu der höchsten Induktion von Interleukin (IL) 6, CCL5, CXCL10, Matrix Metalloproteinase (MMP) 3, MMP13 sowie TLR3 selbst. In RA synovialen Gewebe war TLR3 reichlich vorhanden und vor allem durch RASF in der Intima exprimiert. Des Weiteren wird gezeigt, dass doppelsträngige (ds) RNA von nekrotischen Synovialflüssigkeitszellen freigesetzt wird und dass diese TLR3 auf synovialen Fibroblasten bindet und aktiviert. Diese Resultate lassen vermuten, dass wirtseigene dsRNA für eine anhaltende TLR3 Aktivierung in Gelenken von RA Patienten verantwortlich ist.

In RASF führte die Bindung von TLR3 mit dsRNA zu der Aufregulierung des kürzlich beschriebenen Protein Pre-B-cell colony enhancing factor (PBEF). PBEF ist ein multifunktionales Protein, da es sowohl Nicotinamid Phosphoribosyltransferase wie auch Zytokin und Adipokin Aktivitäten besitzt. Es konnte gezeigt werden, dass die Konzentration von PBEF in Serum und synovialer Flüssigkeit von Patienten mit RA erhöht ist und signifikant mit dem Entzündungsgrad korreliert. Es wird auch gezeigt, dass PBEF selbst eine proinflammatorische und Gelenk zerstörerische Wirkung besitzt: RASF und Monozyten die mit rekombinanten humanen PBEF stimuliert wurden erhöhten die Produktion von Zytokinen,

Chemokinen und matrixdegradierenden Enzymen. Zusätzlich inhibierte PBEF Knockdown in RASF die basale und TLR ligand induzierte IL-6, IL-8, MMP1 und MMP3 Produktion. Zusammengefasst konnte gezeigt werden, dass PBEF einen Marker für den Entzündungsgrad darstellt und ein wichtiges Protein des angeborenen Immunsystems zu sein scheint, in welchem es selbst zu einer chronischen Entzündung beitragen kann.

TLR2, 3 und 4 Liganden induzierten in RASF auch die Expression von p19, eine Untereinheit des heterodimeren IL-23. IL-23 ist ein Zytokin welches aus den Untereinheiten 19 und p40 zusammengesetzt ist, und es wurde angenommen, dass p19 ausschliesslich mit p40 vorkommen kann. Auch wurde vermutet, dass IL-23 in der RA eine Rolle spielen könnte, da es die Proliferation von IL-17 produzierenden T-Zellen fördert. In dieser Arbeit wird gezeigt, dass in RA synovialen Gewebe das Protein p19 durch die synovialen Fibroblasten, die den Gelenksknorpel zerstören, wie auch durch die synovialen Fibroblasten in der Intima exprimiert wird. Jedoch konnte an diesen Stellen kein p40 nachgewiesen werden und auch TLR aktivierte synoviale Fibroblasten in vitro produzierten kein p40. Dementsprechend war in der synovialen Flüssigkeit heterodimeres IL-23 nur sehr schwach detektierbar. Die unterschiedliche Expression der beiden Untereinheiten lässt vermuten, dass entgegengesetzt der bisherigen Annahme, p19 unabhängig von p40 vorkommen kann und es kann spekuliert werden dass p19 in der Pathogenese der RA eine eigene Funktion haben könnte.

Zusammengefasst gibt es viele Hinweise, dass TLR3 und die TLR3 abhängige Signalkaskade in RA eine wichtige Rolle spielen könnte. Diese Daten legen nahe, dass neue Therapieansätze auch die Blockierung von TLR3 und die Blockierung von proinflammatorischen und matrix degradierenden Zytokinen, wie zum Beispiel PBEF, berücksichtigen sollten.

CHAPTER 1 Introduction

Rheumatoid Arthritis

Rheumatoid arthritis is a systemic inflammatory disease leading to joint destruction deformity and loss of function. The most characteristic feature of RA is persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. The joints characteristically involved are the proximal interphalangeal (PIP) and metacarpophalangeal (MCP) joints of the hands, the wrists, shoulders, elbows, knees, ankles, and metatarsophalangeal (MTP) joints. The distal interphalangeal (DIP) joints are generally spared. The severity of RA encompasses varies widely, ranging from mild disease to chronic progressive disease, causing varying degrees of joint destruction and clinically evident extra-articular organ involvement. Extra-articular disease occurs in the majority of patients and is a significant factor in morbidity and mortality. Rheumatoid vasculitis, which can affect nearly any organ system, is seen in patients with severe RA and high titers of rheumatoid factor. Severe extra-articular disease manifestations are generally associated with an increased risk of cardiovascular events (1). In 1987 the American College of Rheumatology (ACR) published a revised set of seven classification criteria for the diagnosis of rheumatoid arthritis (2) (Table I).

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides fo the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Table I: 1987 Criteria for the Classification of Acute Arthritis of Rheumatoid Arthritis. From: American College of Rheumatology, 2007 (www.rheumatology.org).

Epidemiology

RA is the most common form of inflammatory arthritis with a worldwide prevalence of around 1%. However, the occurrence of RA is not the same throughout the world. One large study in Nigeria failed to find a single case, and RA is also rare in China and Indonesia. RA prevalence studies from 14 European countries revealed a possible gradient going from the South (lowest) to the North (highest). Furthermore, the incidence of RA is about 3 times higher in women than in men. For example, the prevalence of RA in men in Finland is reported as 0.6%, in France it is 0.32% while in Italy it is 0.13%. In women the prevalence in the same three countries is 1%, 0.86% and 0.51%. The most recent RA prevalence estimated from Rochester, Minnesota, USA is 1.4% for females and 0.74% for males. Although rheumatoid arthritis may present at any age, the onset of the disease is most frequent in the third to sixth decades (3).

Aetiology

The cause of RA remains unknown, and it is unlikely that a single etiologic factor accounts for all cases of adult RA. A unifying hypothesis for the cause of RA must explain several key features of the disease, namely autoimmunity, chronic inflammation and joint destruction. It is suggested that genetic, environmental, infectious and other so far unknown factors might control the onset, progression, degree, and pattern of inflammation.

Genetics

Genetic factors play an important role and likely account for disease susceptibility and expression. The Class II region of the major histocompatibility complex (MHC) containing the amino acid sequence of the shared epitope (Q/R, K/R, R, A, A) is known to be a major genetic risk factor for RA. The shared epitope sequence extends from position 70 to position 74 on the amino acid chain encoded by HLA-DR1 (DRB1*0101 and 0102), HLA-DR4 (DRB1*0401, 0404 and 0405) and HLA-DR10 (DRB1*1010). The shared epitope is positively charged and contributes to one of the peptide-anchoring pockets of the MHC class II molecules. Therefore, the shared epitope hypothesis suggests that these MHC class II molecules participate in the pathogenesis of RA by selectively binding arthritogenic peptides for presentation to auto-reactive T cells (4). However, the presence of one of the HLA-DR alleles conferring susceptibility to RA is noted in 40% of non-RA individuals and in only

70% of patients with RA, indicating that these alleles are neither necessary nor sufficient to cause the development of RA. It has also been demonstrated that HLA shared epitope alleles are primarily a risk factor for the presence of antibodies to cyclic citrullinated peptide (anti-CCP antibodies) rather than for the development of RA (5).

Several genome screens identified 1p36 as non HLA-susceptibility locus for RA. An association study showed that RA was significantly associated with the TNFR2 196R allele and the TNFR2 196R/R genotype (6). The same locus contains a cluster of genes for peptidyl arginine deiminases (PADs), which catalyze citrullination of arginine residues. A Japanese study showed that eight SNPs in the PADI4 gene were associated with RA (7). However, a study from the UK and a familial study from Europe could not confirm an association with the PADI4 haplotype identified in Japan (8). In addition, other loci, including SLC22A4, RUNX1, PTPN22 and various cytokine and cytokine-receptor loci, such as those encoding for IL-1, IL-10 and IL-18 have been implicated in RA pathogenesis to various degrees in distinct populations (9, 10).

Environment

Genetic risk factors do not fully account for the incidence of RA suggesting that environmental factors might also play a role in the aetiology of the disease. One clearly associated environmental factor is for example cigarette smoking (11). New data support the gene-environment interaction between smoking and the HLA-DRB1 shared epitope in anti-CCP antibody positive but not in anti-CCP negative RA. Given that smoking promotes the citrullination of self proteins, it seems possible that such environmental influences might be directly linked to pathogenic autoantigen-driven responses (12).

Infectious organisms

It has been suggested that RA might be a consequence of an infection with a pathogenic organism in a genetically susceptible host. For example, the glycoprotein gp110 of the Epstein-Barr virus contains the same QKRAA sequence as the shared epitope on HLA-DRB1 alleles. Thus, in certain EBV-infected individuals molecular mimicry may lead to autoimmunity. Other xenoproteins, most notably Escherichia coli DNA J protein, also contain QKRAA and may contribute to a response against self-MHC. But convincing evidence that EBV, other viral or bacterial infections can cause RA has not yet emerged. An alternative hypothesis ascribes the initiation of disease to an activation of the innate immune system in

the synovium. This process involves pattern-recognition receptors on macrophages, dendritic cells, synovial fibroblasts and neutrophils and leads to a non-specific articular inflammation. A local immune response might occur as the synovium permits the influx of lymphocytes, which might recognize a variety of autoantigens (13). In that scenario a non-specific inflammation can lead to local autoimmune responses directed against many articular antigens

Pathogenesis

The main focus of the chronic inflammatory response in the joints of patients with RA is the synovium. The normal synovial tissue is divided into the synovial lining and the synovial sublining layer. The synovial lining is the most superficial cell layer and is in contact with the intra-articular cavity. In the healthy state, the synovium is a thin membrane that encloses the articular space. It is normally composed of two types of synoviocytes, namely macrophages (type A) and synovial fibroblasts (type B), arranged one to two cells thick. The healthy sublining layer contains blood vessels, lymphatics, and nerves and most cells are also fibroblasts and macrophages. Occasionally, lymphocytes, adipocytes and mast cells are as well present in the sublining layer.

The earliest changes found in RA are hyperplasia of the synovial lining cells, injury of the synovial microvasculature with vascular congestion, high endothelial venules and microvascular occlusion as well as cellular infiltrates mainly composed of lymphocytes. This inflammatory stage is usually associated with edema, fibrin exudation and hyperplasia of the superficial lining cell layer. The thickening of the lining layer is the histological hallmark of arthritis. This activated hyperplastic synovial lining layer invades and destroys periarticular bone and cartilage. The major effector cells of cartilage and bone destruction are synovial fibroblasts. Furthermore, in the areas of focal bone erosion resorption lacunae caused by osteoclasts are found. A characteristic of the inflamed synovial sublining is the increased infiltration of T cells, B cells, plasma cells, natural killer cells, dendritic cells, macrophages and occasionally mast cells. Inflammatory cells are often found together in close proximity, either as perivascular infiltrates, particularly around postcapillary high endothelial venules, or as a diffuse infiltrate throughout the synovial sublining. In about 25% of people with RA the lymphoid cellular infiltrate display all characteristics of secondary lymphoid follicles including germinal center-reactions with proliferating B-cells grouped around a network of

follicular dendritic cells. Neutrophils accumulate primarily in the synovial fluid rather than in the synovial tissue but can also be found near the synovium-cartilage junction.

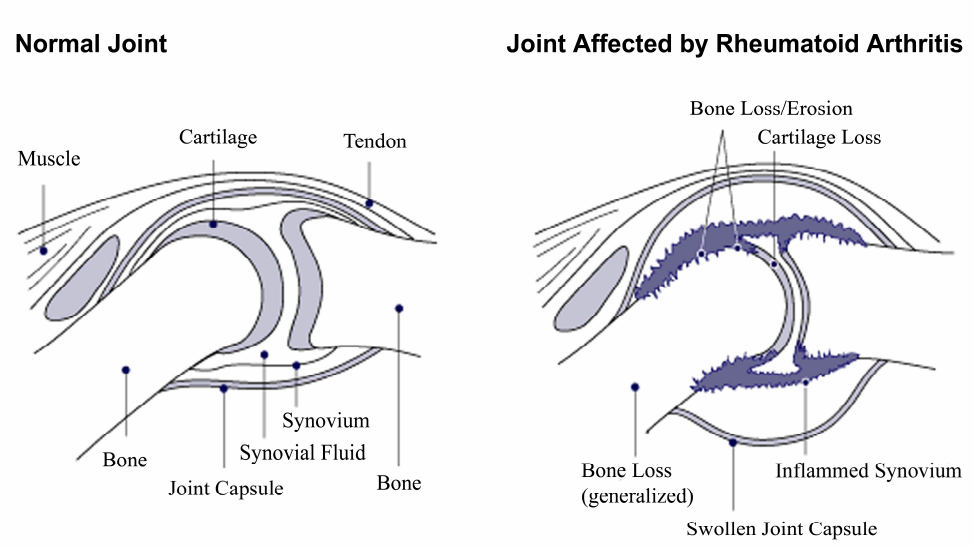

Figure 1: The synovial joint is composed of two adjacent bones, each covered with a layer of cartilage, separated by a joint space and surrounded by synovial tissue and the joint capsule. Rheumatoid arthritis is initially characterized by an inflammatory response of the synovial membrane. As the disease progresses, the inflamed synovium invades and damages the cartilage and bone of the joint. From: National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), NIH Publication No. 04-4179, revised 2004

T-cells

Autoimmunity is classically defined as an antigen-specific immune response to an auto-antigen that leads to disease. For long time it has been favoured that rheumatoid arthritis is a T-cell driven disease as tissue-infiltrating T-cells, mainly CD4+ cells, are a consistent feature of RA. Studies with human synovium engrafted in severe combined immunodeficient (SCID) mouse chimeras, have confirmed that the synovial inflammation is maintained by an antigen-specific T-cell response. Treatment of these mice with T-cell depleting antibodies reduced the production of T-cell derived cytokines and of inflammatory mediators and metalloproteinases produced by synovial macrophages and synovial fibroblasts (14). Furthermore, IL-1 receptor antagonist deficient mice which spontaneously develop arthritis and are therefore used as a RA model, failed to develop arthritis in the absence of mature T cells (15). Conversely, most articular T cells appear quiescent, mitoses are rare and the expression of activation markers such as IL-2 and levels of other T cell derived cytokines are low in the rheumatoid joint. The development of monoclonal antibodies against the T cell surface molecule CD4 has raised

hopes to achieve a major progress in the treatment of RA. However, clinical trials failed to demonstrate the efficacy of anti-CD4-therapy in RA (16). Costimulation of naïve T cells through ligation of CD28 by CD80 or CD86 is perhaps the most important secondary signal to drive T cell proliferation and differentiation. Once activated, T cell upregulate the expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), an inhibitory receptor that has higher affinity for CD80 and CD86, in order to modulate activation. So far, the use of a CTLA-4 immunoglobulin fusion protein (Abatacept) in clinical studies has yielded promising results as a treatment for RA and demonstrated the importance of this cell-cell interaction in immune-mediated diseases (17, 18).

Until recently, T-cell responses were typically classified as either in T-helper (Th) 1 or Th2, based on the relative expression levels of cytokines, especially IFN-γ and IL-4. Although neither Th1 nor Th2 cytokines are present at high levels in the rheumatoid joint, RA had been viewed as a Th1 disease as IFN- γ consistently predominated over IL-4. Evidence from mouse models has questioned the role of Th1 T-cells in RA, and additionally a new T-helper cell subset, characterized by the production of IL-17 (Th17) has been identified (19). Th17 T-cells were demonstrated to be associated with autoimmune joint inflammation, as IL-17 has the ability to induce inflammation and joint destruction when administered in animal models (20). Furthermore, IL-17 levels are elevated in joints of RA patients and synergize with many inflammatory mediators important in joint pathology (21).

T regulatory cells (Treg) have become a major focus of immunologic research in the past decade due to their participation in controlling effector T-cell functions *in vitro* and to their potential for regulating autoimmune inflammatory responses *in vivo* The role of Treg cells in RA is controversial with regard to the number and function of CD4+CD25+ Treg cells. However, most studies have shown that the CD4+CD25+ Treg cells from patients with RA have a defect in suppression of TNF-α and IFN-γ production from CD4+ cells or monocytes, even though they can suppress the proliferation of effector T cells (22, 23).

B-cells

Recently, the B-cell moved into focus as it was reported that the application of anti-CD20 antibody (Rituximab), a B-cell depleting agent, clearly improves clinical symptoms in patients with RA (24). The primary function of a B-cell is the antibody production. In a normal physiological state, the antibody production is a beneficial and required immune

response. This process involves the binding of the antigen and the subsequent activation of the complement system or phagocytosis via Fc receptors. However, if the targeted antigen is a host protein, this mechanism can lead to destruction of tissue and perpetuation of the inflammation. Rheumatoid factors (RF) are the first described human auto-antibodies and they are directed against the Fc region of IgG and are usually of the IgM isotype (25). RF are not specific for RA as they can also be detected in up to 10% of healthy individuals and in patients with chronic bacterial or viral infection, transplanted organs and selected chronic inflammatory diseases. RF may exert several effector functions in the disease process. Deposition of immune complexes containing RFs have been shown in several tissues, and it is likely that activation of the complement cascade by immune complexes contributes to the inflammatory changes in the rheumatoid synovium. However, the evidence for immune complex mediated tissue injury remains indirect and the clinical and pathologic features of RA are not indicative for an immune-complex-mediated disease. Auto-antibodies to citrullinated antigens such as anti-Sa or anti-cyclic citrullinated peptide (anti-CCP) antibodies are dependent on the citrullination of arginine residues posttranslationally generated by peptidylarginine deiminases (26, 27). Anti-CCP antibodies show a remarkable specificity for RA and are strong predictors of disease severity and progression (28). Beside antibody production, B-cells have the potential to present antigens to T-cells. Rheumatoid factor B cells can present any antigen in the context of an immune complex and can activate T cells specific for a variety of foreign antigens. On the contrary, nonspecific B cells, although capable of binding antigen-antibody complexes, fail to present them to T cells (29). Furthermore, it is known that B-cells are also capable to produce proinflammatory cytokines such as TNF, IL-10 and IL-6 (30).

Synovial fibroblasts

In recent years is has become evident that RASF play an important role in the pathogenesis of RA, given that they contribute to the synovial hyperplasia, produce large quantities of inflammatory cytokines and chemokines and mediate cartilage destruction (31-33). Synovial fibroblasts are mainly characterized by the absence of the macrophage specific markers CD68, CD11b, CD14 and CD33 and by the expression of prolyl-4-hydoxylase and vimentin.

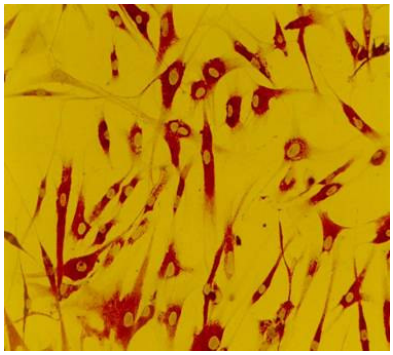

Figure 1: Cultured RASF stained for prolyl-4-hydroxylase . Kindly provided by J. Stanczyk.

Furthermore, synovial fibroblasts are responsible for the production of hyaluronic acid, the major component of normal synovial fluid. RASF in the hyperplastic lining layer and at sites of invasion display numerous features of cellular activation that ultimately result in an aggressive, invasive behaviour. At a morphological level a more round shape and a large pale nucleus with prominent nucleoli characterizes this activated phenotype (34). A central aspect of activated RASF shows the ability to attach to the articular cartilage and to deeply invade into the extracellular matrix. RASF have upregulated adhesion molecules that mediate the attachment to the cartilage. Among the different adhesion molecules, b1 integrins appear to be involved most significantly. The question of whether the activation of RASF is an intrinsic property of these cells or is purely a response to inflammatory cytokines is of crucial importance. In a SCID mouse model the interaction between human synovium and cartilage in the absence of circulating human blood components has been analyzed. In contrast to synovium of patients with osteoarthritis, synovium derived from patients with RA deeply invaded cartilage and expressed mRNA for matrix-degrading enzymes at the site of destruction (35). Furthermore it has been demonstrated that isolated synovial fibroblasts derived from patients with RA but not fibroblasts form patients with OA or fibroblasts of healthy donors, maintain their invasive and destructive capabilities towards healthy human cartilage after several passages in cell culture or when engrafted under the renal capsule of SCID mice (36). These results support the suggestion that the invasive behaviour of RASF is independent of inflammatory cells and cytokines, and therefore intrinsic and persistent.

RASF contribute significantly to joint cartilage and bone degradation through the expression of matrix degrading enzymes such as matrix metalloproteinases (MMPs). In particular RASF in the lining layer and at the sites of cartilage invasion have been identified as the major source of MMPs. The collagenases (MMP-1, 8, -13), the gelatinases A and B (MMP-2 and -9

respectively), the stromelysins (MMP-3, -10, -11), the matrilysins (MMP-7, -26), and the MT-MMPs (membrane-type MMPs) are all expressed at low levels in healthy joint tissue, but their expression is largely increased in arthritic joints (37, 38). In addition to the ability of MMPs to directly degrade cartilage, they are capable of activating other proteases, and thus trigger a cascade of further matrix degrading factors. For example, MT-MMPs have also been shown to activate MMP2 and MMP13. The other group of matrix degrading enzymes are cysteine proteases, which include cathepsins. Cathepsin K has not only been implicated in cartilage but also in bone degradation. Cathepsin K positive RASF are regularly present at sites of cartilage and bone degradation, but cathepsin B and L are also known to be produced by RASF (39, 40)

Besides cartilage degradation, the other typical feature of RA is the thickening of the inflamed synovial lining layer. Whether this synovial hyperplasia is due to the proliferation of RASF or due to an ability to evade apoptosis, is a controversial topic. An indication for rapid proliferation of RASF is provided by the constitutively elevated expression of the transcription factors NF-kB and AP1. In addition, proto-oncogenes that are involved in the regulation of proliferation including myc, myb, ras were also found to be overexpressed in RASF, predominately at sites of invasion into cartilage and bone (39, 40). Furthermore, several soluble factors present in the inflamed joint such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), tumor necrosis factor α (TNF-α), IL-1 and macrophage inhibitory factor (MIF) appear to enhance proliferation (41-44). Alternatively, there is accumulating evidence that an alteration in apoptosis contributes significantly to the synovial hyperplasia in RA. Despite earlier reports based on results from experiments using DNA labelling techniques, suggesting an increased rate of apoptosis among RASF (45), morphological analysis show consistently that apoptosis is a rare event in the rheumatoid synovium (47). Among other cell death receptors, FAS plays a prominent role in mediating apoptosis. RASF are rather resistant to Fas-ligand (FASL) induced apoptosis (46). It is estimated that only about 15% of RASF are susceptible to FAS mediated apoptosis, even though the expression of FAS on RASF is high. Soluble FAS has been detected in synovial fluid of RA patients, and it has been shown to competitively inhibit FAS signalling (48). RASF are also not sensitive to TNF-α mediated apoptosis. TNF-α can activate the apoptotic pathway through the Caspases 3 and 8, but can also initiate pathways leading to the activation of NF-kB. Therefore, in RASF TNF-α induces cell death only after inhibition of NF-kB (49). FLIP and SUMO, two antiapoptotic molecules, can inhibit FAS and

TNF mediated apoptosis (50-52). Levels of FLIP and SUMO are elevated in RA synovial tissues and are expressed predominantly in the synovial lining layer and at sites of cartilage invasion (53, 54). Among other soluble factors present in synovial fluids of RA patients, LIGHT also decreases the susceptibility to FAS mediated apoptosis in RASF (55). In summary, synovial lining hyperplasia in RA appears to be due an interplay of various mitogen and anti-apoptotic stimuli during the course of inflammation.

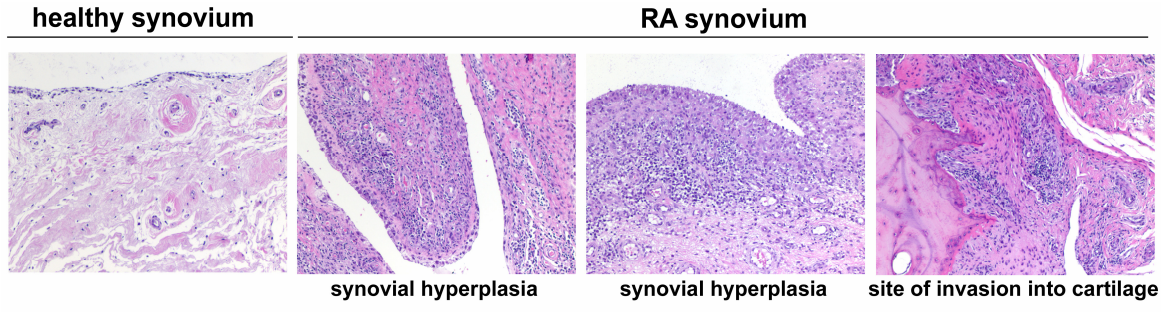

Figure 2: Histology of healthy and rheumatoid synovium. The lining layer in the healthy synovium is only 1-2 cell layers thick. Characteristics of rheumatoid inflammation are hyperplasia of the lining layer and mononuclear infiltrates in the sublining layer. RA synovial fibroblasts invade joint cartilage leading to the destruction of the joint.

Cytokines

In the pathogenesis of RA cytokines play a central role in the perpetuation of synovial inflammation. Cytokines and chemokines are soluble proteins that regulate the immune system and participate in intracellular communications. In general, cytokines secreted by lymphocytes are not prominent in the rheumatoid joint. In contrast, cytokines and chemokines produced by activated macrophages and RASF, such as TNF-α, IL-1, IL-6, IL-8 and CCL-5 are highly abundant in joints of patients with RA.

Several lines of investigation, including experimental and clinical studies, have strongly implicated that TNF-α is of primary importance in the pathogenesis of RA (56). TNF-α stimulates collagenase and PGE2 production by human synovial cells, induces bone resorption, inhibits bone formation and induces cytokine (e.g. IL-6) and MMP production by RASF (57-59). In the collagen induced arthritis mouse model, TNF-α inhibition prevents disease onset and reduces disease severity. Transgenic mice overexpressing human TNF-α spontaneously develop an erosive inflammatory arthritis (60). In clinical studies, therapeutic blockade of TNF-α reached clinical responses in approximately 70% of patients with RA. Thereby similar results were obtained with the TNF inhibitors Infliximab and Adalimumab,

both anti-TNF-α monoclonal antibodies, and Etanercept, a human, soluble, dimeric, TNF type II receptor linked to an IgG1-Fc moiety. (61-63). Notably, intervention with TNF inhibitors early in disease is most effective (64).

IL-1β and TNF-α have a strong overlap of biological effects relevant to inflammation and tissue destruction. Moreover, TNF-α induces IL-1β and IL-1β induces TNF-α in synoviocytes. Similarly to TNF-α, IL-1β induces the production of MMPs, cytokines, chemokines, enhances proliferation and upregulates adhesion molecule in RASF (65). Nevertheless, IL-1β seems to be the stronger inducer of enzyme expression and IL-1 driven animal models of arthritis show rapid and extensive cartilage damage (66). IL-1β further serves as a chemotactic factor for B and T cells. From these studies one could have predicted that blocking IL-1 would also significantly improve disease symptoms in patients with RA. However, only limited efficacy was achieved in clinical trials by blocking IL-1 with recombinant IL-1 receptor antagonist (Anakinra) (67).

IL-6 is a pleiotropic cytokine that has important roles in the regulation of inflammatory responses and hematopoiesis. Overproduction of IL-6 is closely related to the pathological findings in RA, and there are correlations between the elevated IL-6 levels in serum or synovial fluid in clinical and laboratory indices (68). In joints of RA patients synovial fibroblasts are the main producer of IL-6. A direct role for IL-6 in the pathogenesis of RA has been confirmed using several animal models. For example IL-6 deficient mice developed a more modest form of antigen induced arthritis with little evidence of cartilage destruction (69). Inhibition of IL-6 signalling pathways in patients with RA by humanized anti-IL-6 receptor antibodies (Tocilizumab) has so far shown clinical benefit and confirmed the pathological role of IL-6 in the chronic inflammatory disease (70).

The inflammatory chemokines present in RA synovium may contribute to the accumulation of immune cells that are known to express matching chemokine receptors. Activated RASF are a source of chemokines such as IL-8, CCL2 and CCL5. Of interest, levels of IL-8 were found to be significantly higher in clinically inflamed RA joints when compared to clinically uninvolved joints, indicating a role in the development of clinical signs and symptoms. CCL2, the ligand to CCR2, is also highly expressed in RA synovial tissue. Because CCR2 is expressed on T cells, dendritic cells, basophils and natural killer (NK) cells, CCL2 present in

RA synovial tissue can potentially attract all of these cell types. CCL5 is considered to be particularly important as a chemoattractant for T cells and monocytes (71).

Treatment of RA: Challenges and opportunities

The treatment of RA includes anti-inflammatory (NSAIDs = nonsteroid anti-inflammatory drugs, glucocorticoids) and immunosuppressive or immunomodulatory drugs (DMARDs = disease modifying antirheumatic drugs). New DMARDs targeting key cytokines and lymphocytes involved in the pathogenesis of RA have considerably improved therapy of this disease. These so called biologics include TNF-inhibitors, anti-CD20 antibodies targeting B-cells and CTLA-4 antibodies, blocking T-cell co-stimulation. However, a certain proportion of patients show only a partial response or fail to respond. Current clinical data suggests that inhibition of IL-6, IL-15 and perhaps IL-23, IL-18 and IL-17 could also offer therapeutic potential. Choosing the correct target is not easy, as the hierarchical relationships within the synovial cytokine network remains unclear. Therefore, there is a need to identify novel predictive biomarkers for the appropriate selection of targets and for predicting therapeutic responses (72). Furthermore, as synovial fibroblasts play a prominent role in the destruction of articular cartilage, strategies targeting RASF and their effector molecules, such as MMPs and/or cathepsins, should be taken into account. Based on the fact that the treatment of RA is limited and that we can not cure the disease, the present work is designed to search for novel therapeutic targets related to the initiation and/or perpetuation of the disease.

Toll-like Receptors

Toll-like Receptors and the innate immune system

The innate immune system offers a remarkable and rapid defense mechanism against microbial pathogens. An immune response against invading microorganisms is initiated by the recognition of pathogen-associated molecular patterns (PAMP) by germ-line-encoded pattern recognition receptors (PRR). The recognition of PAMPs is mainly attributed to the PRR family of Toll-like receptors (TLR). Initially, the Toll protein was discovered in the fruit fly *Drosophila melanogaster* (73, 74). Flies with a mutated Toll protein, previously known only for its developmental functions, were highly susceptible to fungal infections, suggesting a role in the primary recognition of infectious pathogens (75). The first mammalian homologue of the Toll protein (now termed TLR4) was reported by Medzhitov et al. in 1997: Constitutively activated TLR4 induced the expression of several genes that are involved in inflammatory responses (76). To date, ten human and eleven mouse TLR family members have been identified (77, 78).The activation of TLRs by PAMPs triggers a variety of defense mechanisms depending on the receptor and cell type. This includes the internalization of microbes by phagocytic cells, antimicrobial killing mechanisms such as the production of reactive nitrogen and oxygen species, the production of inflammatory cytokines and chemokines, and the expression of costimulatory molecules. Thereby, in addition to the direct elimination of infectious pathogens, TLRs play a crucial role in linking the innate and the adaptive immunity. The activation of TLRs must be tightly regulated since a weak response may leave the host susceptible to infections, whereas a strong response may lead to lethal systemic inflammation or autoimmunity. A direct link between defects in TLR signalling and a specific disease has not been firmly established so far. However, there is evidence for a contributory role of TLR signalling in various autoimmune diseases.

Toll-like receptor signalling

All TLRs are type 1 transmembrane receptors and recognize distinct ligands through their leucine-rich repeats in the extracellular domain. The cytoplasmic portion of the receptors include a conserved cytoplasmic motif, the Toll/interleukin (IL)-1 receptor (TIR) domain(79). The TIR domain of TLRs is homologous with the respective domain of the interleukin 1 receptor (IL-1R) and the cytoplasmic adaptor protein family. The TIR domains of the adaptor

proteins interact with the TIR domains of TLRs or IL-1R, thereby triggering the activation of downstream protein kinases and different transcription factors. To date, five different adaptor proteins are known to be involved in TLR signalling: MyD88 (myeloid differentiation factor 88), MAL/TIRAP (MyD88-adaptor-like/TIR associated protein), TRIF (TIR domain-containing adaptor-inducing IFN-β), TRAM (Toll-receptor-associated molecule) and SARM (sterile alpha and HEAT/Armadillo motif protein). MyD88 is the best characterized adaptor protein. Its structure includes a N-terminal death domain and a C-terminal TIR domain. MyD88 is essential to the signalling pathways of all TLR family members, with the exception of TLR3 (80-82).

MyD88 dependent pathway

After binding of TLR ligands to the corresponding receptor, MyD88 recruits IL-1 receptor associated kinases (IRAKs) to TLRs through interaction of the death domains of both molecules. To date, four different IRAK-like molecules have been identified: two active kinases, IRAK-1 and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-M. Phosphorylated IRAK1 and IRAK4 dissociate from the receptor complex and consecutively bind to TNF receptor associated factor 6 (TRAF6) (83, 84). Associated TRAF6 leads to the activation of the IκB kinase (IKK) complex, which contains two catalytic subunits IKK-α and IKK-β and a scaffold protein termed NEMO/IKK-γ. The IKK complex then induces the activation of the NF-κB transcription factor, as well as the MAP kinases, namely ERK, p38 and JNK. IRAK-M negatively regulates TLR signalling pathways by preventing the dissociation of IRAK-1 and IRAK-4 from the receptor complex. Recently, it was demonstrated, that TLR2 and TLR4 signalling requires the interaction of TRAF6 with MAL, whereby MAL is suggested to link TLR2 and TLR4 directly with TRAF6 mediating the subsequent NF-κB activation (85).

MyD88 independent pathway

Analysis of MyD88 deficient mice showed that TLR3 and TLR4 are capable of inducing certain signalling pathways independently of MyD88 (86, 87). In addition to the activation of NF-κB and MAP kinases, the hallmark of the MyD88 independent pathway is the activation of the transcription factor IRF-3 leading to the induction of type I interferon (IFN) expression (88, 89). In contrast to TLR3, activated TLR4 has the ability to induce MyD88 dependent and independent pathways. Whereas the adaptor protein TRIF interacts with TLR3 directly, the interaction with TLR4 is dependent on the adaptor protein TRAM. The signalling cascade

downstream of TRIF leading to IRF-3 activation involves the noncanonical IκB kinase homologs IKK-ε and TANK-binding kinase-1 (TBK-1). Activated TBK-1 forms a complex with TRIF and IRF-3, leading to the expression of IFN-α/β and interferon responsive genes (90). Recently, it was shown that Rho GTPase Rac-1 and p21-activated kinase (PAK-1) play a role in IRF-3 activation acting upstream of IKK-ε (91). In addition, TRAF6 interacts physically and functionally with TRIF. The resulting complex triggers the activation of NF-κB, leading to proinflammatory cytokine production (92). Moreover, receptor interacting protein (RIP)-1 and RIP-3, interact with TRIF through the RIP homotypic interaction motif (RHIM) found in the C-terminal region of TRIF. RIP-1 is essential for the TRIF dependent NF-κB activation, whereas RIP-3 interrupts TRIF signalling by preventing the association between RIP-1 and TRIF (93).

Taken together, upon binding of distinct microbial components, the interaction between individual TLRs and their corresponding adaptor proteins leads to differential activation of signalling pathways.

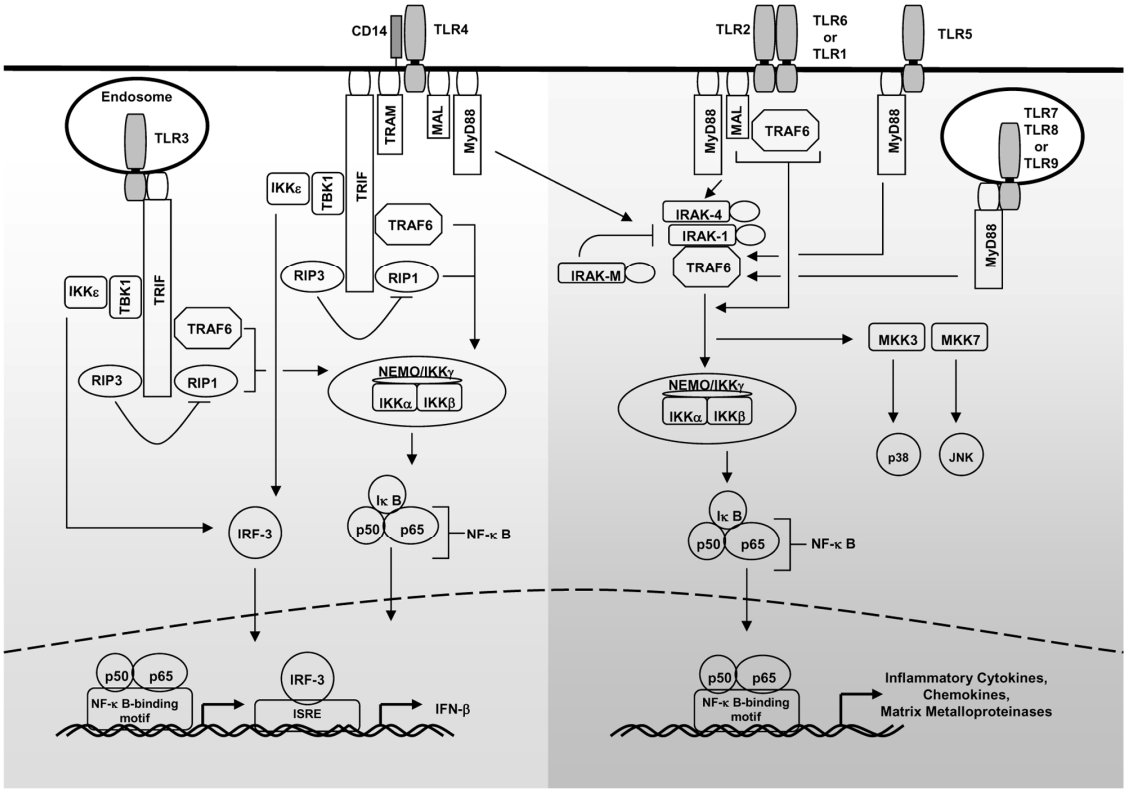

Figure 3: Toll-like receptor signalling. From: Brentano et al., Cell Immunol. 2005 Feb;233(2):90-6.

TLR Ligands

According to their corresponding ligands, TLRs can be categorized in two main groups: TLRs 1, 2, 4 and 6 are receptors for lipid-based PAMPs (diacylated and triacylated lipoproteins; LPS) while TLRs 3, 7, 8 and 9 are receptors for nucleic acid–based PAMPs (dsRNA, ssRNA, CpG DNA). Besides the main categories, TLR5 represents the receptor for flagellin, the major structural protein of the flagella of gram-negative bacteria. Murine TLR11 recognizes uropathogenic bacterial lysates whereas specific ligands for TLR10 are currently unknown.

Lipid-based TLR ligands

In 1998, lipopolysaccharide (LPS), a glycolipid component of the outer membrane of gram-negative bacteria, was identified as the main ligand for TLR4 (94). LPS plays a central role not only in eliciting inflammatory responses, but also in the pathogenesis of septic shock during gram-negative bacterial infections. Other molecules recognized by TLR4 are lipid A analogs, taxol and mycobacterial components. Additionally, various host derived molecules binding to TLR4 could be demonstrated. These endogenous ligands include fibrinogen (95), surfactant protein-A (96), fibronectin extra domain A (97), heparan sulfate (98), soluble hyaluronan (99), β-defensin 2 (100), high mobility group box 1 protein (HMGB1) and heat shock proteins, including Hsp60, Hsp70, Hsp22 and Gp96 (101-105).

TLR2 ligands are the most diverse group among all TLR ligands due to the heterodimerization of TLR2 with either TLR1 or TLR6. TLR1/TLR2 heterodimers recognize native mycobacterial lipoprotein and several triacylated bacterial lipoproteins, such as the synthetic structure S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH trihydrochloride (Pam3Cys) and meningococcal lipoproteins. TLR6/TLR2 heterodimers recognize diacylated bacterial components including the mycoplasma lipoprotein (MALP) and peptidoglycan (PGN) (106) (107, 108). The heat shock proteins Hsp60, Hsp70, Gp96 and HMGB1, already mentioned to be TLR4 ligands, were identified to function as host derived ligands for TLR2 as well. Several endogenous molecules binding to TLR4 or TLR2 are capable of inducing the release of proinflammatory cytokines from the monocyte-macrophage system and the activation and maturation of dendritic cells. However, recent evidence has been presented suggesting that the reported cytokine effect of heat shock proteins may be a result of contamination with LPS (109). Therefore additional investigations

are awaited to clearly distinguish between specific effects of endogenous TLR ligands and contamination with microbial products.

Nucleic acid based TLR ligands

Generally, the TLRs recognizing nucleic acid based PAMPs are located in the endosome. Several studies have shown that inhibitors of endosomal acidification, such as chloroquine and bafilomycin A1, inhibited TLR3-, TLR7-, and TLR9-mediated signalling pathways, indicating that these signalling pathways require acidification and maturation of endosomes (110-112).

TLR3 has been reported to be a receptor for double stranded RNA (dsRNA). Many viruses synthesize dsRNA during their replicative cycle, suggesting that TLR3 might be a key molecule in the initiation of anti-viral host defense. TLR3 also recognizes polyriboinosinic:polyribocytidylic acid, poly(I:C), a synthetic analog of dsRNA. Moreover, it could be shown that mRNA acts as an endogenous ligand for TLR3. Kariko et al. proposed that short segments of dsRNA within mRNAs allow signalling through TLR3 (113). ssRNA viruses and GU-rich ssRNAs are recognized by TLR7 and TLR8, indicating that ssRNA represents a physiological ligand for TLR7 and TLR8 (114-116). Additionally, it was shown that TLR7 is activated by low molecular anti-viral compounds including various imidazoquinolines (imiquimod and R-848) and guanosine analogs (Loxoribin) (117). Peritoneal macrophages from wild-type mice stimulated with imiquimod or R-848 produced TNF-α, IL-6 and IL-12, whereas TLR7-deficient macrophages produced no detectable amounts of these cytokines in response to either compound (115). R-848 also activates TLR8 in humans but not in mice, suggesting that mouse TLR8 is not functional.

TLR9 is the main receptor for bacterial unmethylated deoxy-cytidylate-phosphate-deoxy guanylate (CpG) DNA (118, 119). Unmethylated CpG-dinucleotide-containing sequences are found much more frequently in bacterial genomes than in vertebrate genomes. Unmethylated CpG oligodeoxynucleotides have immunostimulatory activities including the capability to induce B cell proliferation and to activate macrophages and dendritic cells.

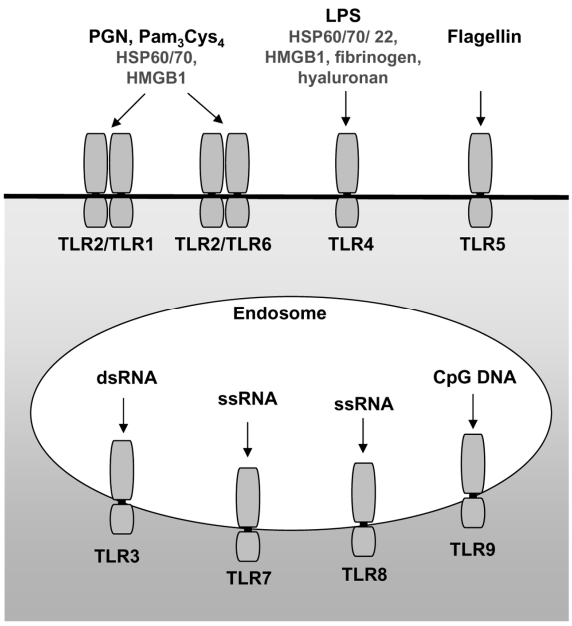

Figure 4: Toll-like receptors and their corresponding ligands

TLR expression in different cellular subsets

The identification of combinatorial sets of TLRs on different cellular subsets is critical to the understanding of pathogen-host interaction as they are associated with the recognition of specific sets of potential ligands (120, 121). Monocytes express high levels of TLR1, 2, 4, 5 and 8, low levels of TLR6 and undetectable levels of TLR3, 7, 9 or 10. Immature myeloid DCs express high levels of TLR1, 2 and 3, low levels of TLR5, 6, 8 and 10, and undetectable levels of TLR4, 7 and 9. In marked contrast plasmacytoid pre-DCs express high levels of TLR7 and 9, low levels of TLR 1, 6 and 10 but undetectable levels of TLR2, 3, 4, 5 and 8. The remarkable differences among monocytes, immature myeloid DC's and plasmacytoid pre-DCs, in their TLR repertoire expression, suggests that cells have evolved to recognize different pathogens. During the differentiation of immature to mature DC's a progressive downregulation of TLRs occurs. That observation indicates a functional switch of the DC lineage from antigen recognition to antigen presentation, thus priming naïve T-cells and instructing them to differentiate into appropriate effector T cells. Certain TLRs are also expressed in T-cells although at low levels. Peripheral blood T cells express TLR1, 2, 3, 5 and 9 whereas regulatory T cells (CD25high, CD4+) cells express TLR2, 5 and 8 (122). The best known TLR member expressed in human B cells is TLR9. In fact, CpG motifs were discovered as TLR9 ligands based on B cell stimulation (123). Besides TLR9, human

peripheral blood B cells express high levels of TLR1, TLR6, and TLR10, intermediate levels of TLR7, and low levels of TLR2 and TLR4. Polymorphonuclear cells (PMN) express TLR1, 2, 4, 5 and 6 (124).

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	ref
Monocytes	++	+++		+++	++	+		++			120
mDCs	++	++	+++		+	+		+		+	120/121
pDCs	+					+	+++		+++	+	120/121
B- cells	++	+		+		++	+		+++	++	120/124
T- cells	+	+	+		++				+		120/122

Table II: Expression of TLRs mRNA in cellular subsets of human peripheral mononuclear cells.

The role of TLR signalling in arthritis

It has been suggested that the innate immune system might be involved in early events of an inflammation leading to clinically active and sustained disease. Potential mediators in these events are synovial macrophages, dendritic cells and synovial fibroblasts, which can all detect and react to danger signals without the support of effector cells of the adaptive immune system. Recognition of invading microorganisms by PRR such as TLRs results in the activation of genes encoding proinflammatory cytokines and chemokines, which set the stage for a local inflammatory reaction. In addition, TLR signalling leads to upregulation of costimulatory molecules by antigen presenting cells, facilitating the subsequent activation of the adaptive immune system. The innate immune system is therefore controlling adaptive immune responses via the provision of the second signal to T cell stimulation (125). Because of this important role of the innate immune system it has been postulated that a dysregulation of innate immune recognition of pathogens may be associated with autoimmunity.

Polymorphisms

Studies of single nucleotide polymorphism (SNP) in genes encoding TLRs or their signalling molecules have provided insights into TLR-related immune disorder. The best studied TLR polymorphism is the SNP Asp299Gly in the human TLR4 gene (126). This polymorphism was found to be associated with impaired airway responses and hyporesponsiveness to inhaled LPS challenge (127). Additionally, Asp299Gly TLR4 displays a lower response to

LPS in vitro and increases the risk of gram-negative bacterial infection (128). In inflammatory bowel diseases, such as Crohns disease and ulcerative colitis, an association with the TLR4 polymorphism Asp299Gly was reported (129). The Asp299Gly variant was also shown to be associated with decreased susceptibility to RA, but not to influence disease severity and/or outcome (130). However, an other study demonstrated no association of the same TLR4 polymorphisms with the susceptibility or severity of RA (131). Similarly, recent reports showed no influence of TLR4 polymorphisms on the susceptibility of juvenile idiopathic arthritis or ankylosing spondylitis (132). SNPs in the TLR2 gene and in its promotor have also been reported. Two independent studies showed that the TLR2/-16 934 SNP is a major gene related to asthma in children (133). However in RA no association with the TLR2 polymorphism has been reported so far. Therefore, although several TLR polymorphisms have been reported to be associated with autoimmune disease, no evident TLR mutation has been identified to be associated with RA.

Animal models

In spite of the lack of sufficient evidence for a linkage of TLR mutations and autoimmune diseases there are experimental results suggesting an involvement of TLR signalling pathways in the pathogenesis of arthritis. Classical animal models of arthritis such as adjuvant arthritis or streptococcal cell wall arthritis are dependent on the activation of the innate immune system by TLR ligands. Mice deficient for the adaptor molecule MyD88 did not develop streptococcal cell wall induced arthritis, and TLR2 deficient animals exhibited a significantly reduced severity of the arthritis (134). Moreover, the injection of either staphylococcal peptidoglycan, CpG DNA or dsRNA into joints of mice results in a self-limited form of arthritis (135-137). These studies suggest, that the availability of TLR ligands might be sufficient to initiate arthritis in a susceptible host. TLR signalling may not only be important early in the pathogenesis of arthritis, but also when the adaptive immune system is activated. In the model of murine antibody transferred arthritis K/BxN, a form of arthritis which is dependent on IL-1, a recent study demonstrated that LPS can substitute for IL-1 and that TLR4 deficient mice have a decreased severity of the arthritis (138). Recently, it has also been demonstrated that inhibition of TLR-4 suppresses the severity of experimental arthritis and results in lower IL-1 expression in arthritic joints (139). Therefore, TLR signalling has an important influence on the development of joint inflammation, even in a situation when T-cell and B-cell activation has already occurred and arthritogenic antibodies are present. Furthermore, in a seminal study Marshak-Rothstein and colleagues have demonstrated in a

transgenic mouse model, that chromatin-containing immune complexes can activate B-cells to produce rheumatoid factor auto-antibodies by synergistic engagement of the B-cell receptor and TLR9 (140). Apart from explaining why auto-antibodies with specificity for nuclear antigens predominate in certain rheumatic diseases, the study established a critical role for endogenous TLR ligands in the dysregulation of the adaptive immune system characteristic of autoimmunity.

TLR ligands in the rheumatoid joint

In septic arthritis, TLR ligands are present in high amounts, however, with antibiotic treatment inflammation eventually will subside. In rheumatoid arthritis, TLR ligands of microbial origin, peptidoglycans and double stranded DNA, have been detected in joints of patients (141). Whether these TLR ligands lead to chronic stimulation of the innate immune system is unclear. Alternatively, host derived endogenous ligands might specifically activate TLRs and in the absence of regulatory mechanisms cause a pathological immune response. Many endogenous ligands for various TLRs have been described, some of which can be found in joints of patients with arthritides. For example extracellular matrix (ECM) components, such as fibronectin, hyaluronic acid and heparin sulphate, are produced in response to tissue injury and inflammation and might act as endogenous TLR2 and TLR4 ligands in joints of patients with RA. Furthermore extravascular deposition of fibrinogen and fibrin clots might further trigger the TLR signalling cascade via TLR4. Extracellular heat shock proteins and HMGB1, both endogenous ligands or TLR2 and TLR4, have previously been implicated in the pathogenesis of arthritis (142, 143). Necrotic cells, which are usually abundant in damaged tissue, e.g. in the arthritis joint, were shown to liberate heat shock proteins as well as HMGB1 into the extracellular milieu. Regarding endogenous ligands of TLR9, blood serum DNA was found to be considerably enriched with fragments of ribosomal repeats containing immunostimulatory CpG-motifs in patients with RA (144). Taken together, there is ample evidence for the presence of exogenous as well as endogenous TLR ligands in autoimmune diseases, including rheumatoid arthritis.

TLR expression and function in the rheumatoid joint

Our laboratories has been leading in the detection of TLRs in the rheumatoid joint (145-147). Analysis of synovial tissues of patients with RA revealed TLR2 expression in the synovial lining on fibroblasts as well as on macrophages (145). Synovial fibroblasts cultured in vitro upregulate the expression of TLR2 upon stimulation with IL-1 and the TLR2 ligand

peptidoglycan. Detailed analysis of gene expression following activation of TLR2 with peptidoglycan revealed induction of proinflammatory cytokines such as IL-6, tissue destructive matrix metalloproteinases and adhesion molecules (146). In addition, high amounts of chemokines are produced upon stimulation via TLR2 in vitro (147). The in vitro expressed cytokines and chemokines corresponded with elevated concentrations found in synovial fluid of patients with rheumatoid arthritis. Activation of TLR2 and TLR4 signalling pathways in RASF further induces RANKL expression promoting the differentiation of osteoclasts in RA synovium (148). Besides synovial fibroblasts, monocytes/macrophages are the main sources of cytokines and chemokines. An elevated frequency of CD16+ monocytes in the peripheral blood of patients with RA was reported. These CD16+ cells expressed higher levels of TLR2 as compared to CD16- monocytes and produced large amounts of TNF-α, IL-1, IL-6 and IL-8 in response to TLR2 agonists. Interestingly, in the synovial tissue CD16+ macrophages were mainly localized in the lining layer (149). TLR activated dendritic cells might also be implicated in the pathogenesis of RA, as they represent the major antigen presenting cells. Of note, TLR ligands induce maturation of dendritic cells leading to the production of various proinflammatory mediators. Interestingly TLR3 and TLR4 potently acted in synergy with TLR7, TLR8 and TLR9 (150). A recent report demonstrated that macrophage migration inhibitory factor (MIF) production by dendritic cells is also regulated by TLRs and increased during RA (151). These results suggest that TLR signalling pathways are operative in the course of the arthritis although the causal relationship is not clear.

Objective

Rationale

There is evidence for an activation of TLR signalling pathways in rheumatoid arthritis. For example we found elevated TLR expression in patients with RA compared to patients with OA. However, it is not clear whether TLR activation represents a primary or a secondary phenomenon related to the ongoing inflammation in the joints. We hypothesize, that the induction of inflammation in the synovium might be due to an unspecific activation of the innate immune system. In this process recognition of infectious agents by TLR might lead to the induction of articular inflammation. Thereby it is crucial that the activation of TLRs on synoviocytes is tightly regulated, since a weak response may leave the host susceptible to infections, whereas a strong response may lead to sustained inflammation. In patients with RA, TLRs might be involved in the switch from transient to persistent inflammation. Our hypothesis is, that chronic inflammation in the joints of patients with RA leads to an abundance of endogenous ligands. These ligands might further activate TLR signalling leading to autoimmune inflammation via enhancement of a positive feedback loop mechanism.

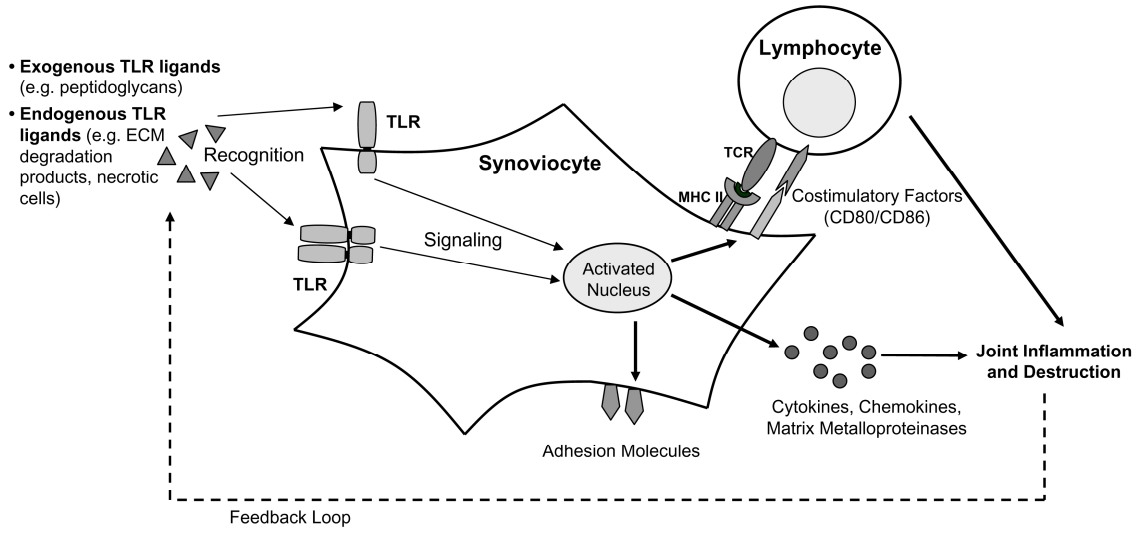

Figure 5: Possible involvement of Toll-like receptors in the pathogenesis of RA. Synovial fibroblasts and macrophages activated by the recognition of microbial components or endogenous ligands by TLRs upregulate the expression of proinflammatory cytokines, chemokines, and tissue destructive enzymes. In a hypothetical feedback loop, endogenous TLR ligands generated by the inflammatory processes may result in chronic stimulation of synovial cells. From: Brentano et al., Cell Immunol. 2005 Feb;233(2):90-6.

Aims

Synovial fibroblasts play a key role in the joint destruction in RA and produce large amounts of cytokines and chemokines (33). The characteristic aggressive and invasive behaviour of RASF might be a result of persistent activation of TLR signalling pathways. In previous reports by our laboratory, the expression of functional TLR2 on RASF has been documented (145). However, the expression level of TLR2 and the other known TLRs has not been determined so far. In **Chapter 2** we established a quantitative expression profile of the currently known TLRs (TLR1-10) in RASF and compared the relative expression levels to osteoarthritis (OA) synovial fibroblasts as well as to normal synovial fibroblasts. Furthermore, by stimulation experiments in vitro, the functionality of the different TLRs in RASF and OASF was investigated. As TLR3 was highly expressed by RASF, we analyzed in **Chapter 3,** whether TLR3 might play a role in the pathogenesis of RA. We searched for possible endogenous TLR3 ligands that might be present and responsible for sustained TLR3 activation in joints of patients with RA. In order to investigate TLR3 dependent gene regulation we performed substractive hybridization between untreated and TLR3 ligand treated RASF. We found Pre-B-cell colony enhancing factor (PBEF) to be upregulated in TLR3 activated RASF. In **Chapter 4,** we investigated the expression, regulation and function of PBEF in RA. Another gene which was highly upregulated in RASF following stimulation with TLR ligands was the IL-23 subunit p19. In **Chapter 5** we studied the expression and regulation of heterodimeric IL-23 in the rheumatoid synovium. In summary, we examined whether targeting TLRs, TLR signalling molecules and especially cytokines downstream of TLR activation might be a promising approach to treat rheumatoid arthritis.

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CHAPTER 2

TLR expression in synovial fibroblasts

Objective

In recent years, we have shown that activation of Toll-like receptor (TLR) 2 pathways in rheumatoid arthritis synovial fibroblasts (RASF) leads to the production of various effector molecules. In particular, stimulation of RASF with the TLR2 ligands peptidoglycan or bacterial lipoprotein, induced the production of IL-6, MMP1, 3 and 9, as well as the secretion of various chemokines, such as GCP2, MCP1 and CCL5. In contrast, stimulation of RASF with TLR9 ligands, such as CpG oligonucleotides, had no effect on the production proinflammatory or destructive effector molecules. These results suggested the expression of TLR2 but not TLR9 on RASF. The expression of other TLR family members and their functionality have not been assessed so far. Therefore, we established a quantitative expression profile of the currently known TLRs (TLR1-10) in RASF and compared their relative expression levels to osteoarthritis (OA) synovial fibroblasts as well as to normal synovial fibroblasts (NSF) and skin fibroblasts. Furthermore, by stimulation experiments in vitro we investigated the functionality of the different TLRs in RASF, OASF and skin fibroblasts.

Material and Methods

Patients and tissue preparation. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and OA (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASF and OASF were isolated from synovial tissues, digested by collagenase, and used after passages 4 to 8 as described. All RA patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) criteria for the classification of RA.

Cell culture. RASF and OASF were cultured in DMEM (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum. RASF and OASF were stimulated with the following agents for 24h: palmitoyl-3-cysteine-serine-lysine-4 (bLP, 300 ng/ml, Invivogen). Polyinosinic-polycytidylic acid (poly(I-C), 20 ug/ml; Invivogen, San Diego, CA), lipopolysaccharide from E. coli (LPS, 100 ng/ml; List Biologicals, Campbell, CA), flagellin (500ng/ml, Invivogen), loxoribine (100uM, Invivogen), CpG PS2006 (CpG, 1ug/ml; Microsynth).

Real-time PCR. Quantification of specific TLR mRNA was performed by Taqman (TLR1-9) and SYBR green (TLR10) Real-time PCR. Primer sequences are listed in in table III. Complementary DNA (cDNA) was generated by reverse transcription of total RNA using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). As negative controls non reverse transcribed samples were used. For quantification of mRNA levels, single-reporter Real-time PCR was performed using the ABI Prism 7700 Sequence Detection system (Applied Biosystems, Rotkreuz, Switzerland). Eukaryotic 18S rRNA levels, measured with a pre-developed primer/probe system (Applied Biosystems, Rotkreuz, Switzerland) served as endogenous control for relative quantification. The difference between the values of comparative threshold cycles (Ct) of the tested gene and 18S (dCt) was used to calculate changes of relative expression (ddCt) after stimulation following the formula ddCt = dCt (sample stimulated) - dCt (sample unstimulated). X-fold regulation was calculated using the expression 2-ddCt. Only samples with a difference of at least 4 cycles between cDNA and non reverse transcribed RNA samples were considered for calculations.

	forward primer	reverse Primer	probe
TLR1	CAGTGTCTGGTACACGCATGGT	TTTCAAAAACCGTGTCTGTTAAGAGA	TGCCCATCCAAAATTAGCCCGTTCC
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT	TCCATCCCATGTGCGTGGCC
TLR3	CCTGGTTTGTTAATTGGATTAACGA	TGAGGTGGAGTGTTGCAAAGG	ACCCATACCAACATCCCTGAGCTGTCAA
TLR4	CAGAGTTTCCTGCAATGGATCA	GCTTATCTGAAGGTGTTGCACAT	CGTTCAACTTCCACCAAGAGCTGCCT
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAACCACCACCATGATGAG	CCAGGGCAGGTGCTTATCTGACCTTAACA
TLR6	GAAGAAGAACAACCCTTTAGGATAGC	AGGCAAACAAAATGGAAGCTT	TGCAACATCATGACCAAAGACAAAGAACCT
TLR7	TTTACCTGGATGGAAACCAGCTA	TCAAGGCTGAGAAGCTGTAAGCTA	AGAGATACCGCAGGGCCTCCCG
TLR8	TTATGTGTTCCAGGAACTCAGAGAA	TAATACCCAAGTTGATAGTCGATAAGTTTG	TGATTTCCAGCCCCTGATGCAGC
TLR9	GGACCTCTGGTACTGCTTCCA	AAGCTCGTTGTACACCCAGTCT	ACGATGCCTTCGTGGTCTTCGACAAA
TLR10	CTGATGACCAACTGCTCCAA	AGTCTGCGGGAACCTTTCTT	-

Table III: Primer sequences used for Real-time PCR

Enzyme linked immunoabsorbant assay (ELISA). IL-6 was detected by ELISA with the OptEIA® Kit (BD Pharmingen, San Diego, CA, USA).

Results

Quantitative expression of TLR 1-10 in synovial fibroblasts. To define the basal expression pattern of all currently known human TLRs in RASF we performed quantitative Real-time PCR for TLR1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. The highest basal mRNA expression was found for TLR3. Also TLR4 mRNA was expressed at high levels. Intermediate levels were found for TLR1, 2 and 6 mRNA and low levels were detected for TLR5. mRNA expression levels for TLR7, 8, 9 and 10 were under the detection limit in unstimulated RASF and could also not be induced by various stimuli (data not shown). When we compared the basal levels of TLR expression in RASF to OASF and normal SF (NSF), levels for TLR2 were more abundant in RASF, and the median expression of TLR3 was also slightly higher in RASF compared to OASF and NSF. However, the differences did not reach statistical significance. TLR1, 4 and 6 were expressed to similar levels in RASF, OASF and NSF. In skin fibroblasts the same TLRs were detected as in synovial fibroblast and no statistical difference was observed concerning their expression levels. In summary, we show that synovial fibroblasts as well as skin fibroblasts express TLR1-6 but not TLR7-10 and mRNA for TLR3 was found to be most abundant.

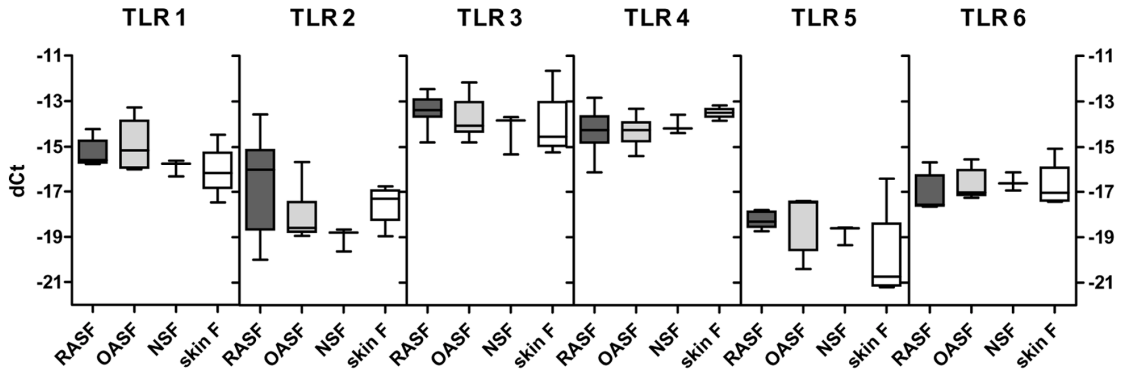

Figure1: Basal mRNA expression levels for TLR1, 2, 3, 4, 5 and 6 in RASF (n=8), OASF (n=6), NSF (n=3) and skin fibroblasts (n=4), determined by quantitative Real-time PCR. The gene expresseion levels were determined as described in Materials and Methods and normalized to the level of 18S rRNA. Since all primer/probe combinations amplify with essentially equal efficiencies, TLR expression levels are directly comparable. Lines within the boxes represent the median, the boxes represent the 25th and 27th percentiles, and the lines outside the boxes correspond to the minimum and maximum values.

Activation of TLR signaling pathways by corresponding TLR ligands. Since we found that TLR1, 2, 3, 4 and 5 are differentially expressed in synovial fibroblasts we analyzed whether they also differ in their property to transmit the induction of pro-inflammatory

cytokines, such as IL-6. We stimulated RASF and OASF with optimal concentrations of the TLR2/1 ligand peptidoglycan, the TLR2/6 ligand bLP, the TLR3 ligand poly(I-C), the TLR4 ligand LPS, the TLR5 ligand flagellin, the TLR7 ligand loxoribine or with the TLR9 ligand CpG and determined the levels of IL-6 in culture supernatants by ELISA. The basal levels of IL-6 were higher in RASF compared to OASF cultures (RASF 4.8 ± 2.5 ng/ml IL-6, OASF 3.18 ± 2.3). Stimulation with loxoribine or CpG oligonucleotides did not enhance IL-6 production in RASF nor in OASF, confirming the absence of TLR7 and 9 in synovial fibroblasts. In contrast, the TLR2, 3, 4 and 5 agonists upregulated IL-6 production significantly in RASF as well as in OASF. Poly(I-C) was the most potent inducer of IL-6, followed by LPS, flagellin, bLP and PGN. The absolute levels of IL-6 were higher in TLR ligand stimulated RASF than in TLR ligand stimulated OASF cultures, but the difference did not reach statistical significance. In accordance, the relative upregulation of IL-6 production by TLR ligands was similar in RASF and OASF. Skin fibroblasts stimulated with TLR ligands also upregulated IL-6 production, but produced significantly less IL-6 after TLR2 and 3 activation when compared to RASF. In general we show functional expression of TLR1-6 in synovial and skin fibroblasts. However, synovial fibroblasts derived from patients with RA produced the highest amounts of proinflammatory IL-6 following TLR activation.

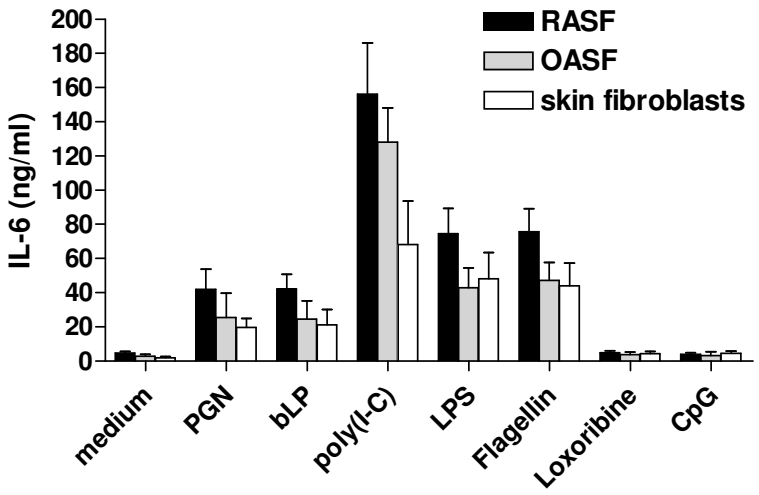

Figure 2: IL-6 production in untreated and TLR ligand stimulated RASF (n=9), OASF (n=4) and skin fibroblasts (n=4). 24h following stimulation, IL-6 concentration was measured in the culture supernatant by ELISA. The mean levels of IL-6 ± SEM are indicated.

Production of matrix-degrading enzymes after stimulation with TLR ligands. As matrix-degrading enzymes, such as matrix metalloproteinases, play a major role in the destruction of articular cartilage, we analyzed whether TLR agonists have the potential to induce MMP3 and

13 in RASF, OASF and skin fibroblasts. Fibroblast cultures were stimulated for 24h with the TLR2 ligand bLP, the TLR3 ligand poly(I-C) or with the TLR4 ligand LPS. The induction of MMP3 and 13 was determined by taqman Real-time PCR. In RASF, poly(I-C) induced MMP3 mRNA expression more than 60 fold and MMP13 more than 90 fold. bLP and LPS also induced MMP3 and 13 mRNA expression in RASF but to a much lesser extent. In OASF similar results were obtained (data not shown). In opposition, skin fibroblasts did not significantly upregulate MMP3 nor MMP13 mRNA expression following TLR activation.

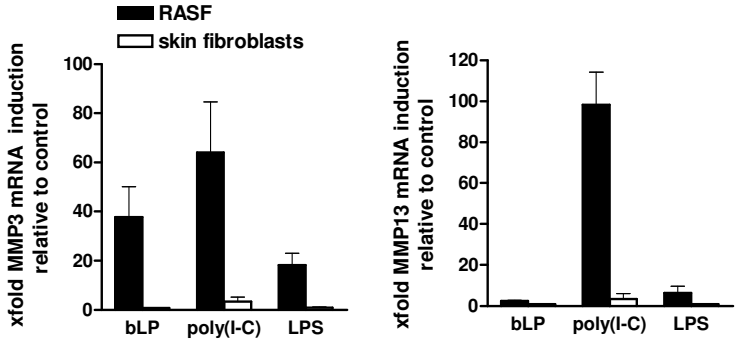

Figure3: MMP3 and MMP13 mRNA induction by TLR ligands in RASF (n=6) and skin fibroblasts (n=3).. The induction of MMP mRNA was determined by Real-time PCR 24h after stimulation with the indicated TLR ligands. The mean upregulation of MMP3 and 13 ± SEM is indicated.

Conclusion

We show functional expression of TLR1, 2, 3, 4, 5 and 6 in synovial fibroblasts. Our data show that TLR3 is the most abundant TLR expressed in fibroblasts and activation of TLR3 leads to the most marked induction of IL-6, MMP3 and MMP13. This result was surprising as the expression of TLR3 has been suggested to be restricted to myeloid dendritic cells. There was no significant difference in the expression level of TLRs between RASF, OASF, NSF or skin fibroblasts. However, functional differences between synovial and skin fibroblasts have been observed. In response to TLR activation, synovial fibroblast produce significantly more IL-6 and highly upregulate MMP3 or MMP13 mRNA expression. In general our results suggest that activation of TLRs expressed on resident synovial fibroblasts contribute to chronic inflammation and joint destruction. In particular, the results strongly support further investigation of TLR3 in the pathogenesis of RA.

CHAPTER 3

RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3

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Abstract

Objective. To assess the expression of Toll-like receptor 3 (TLR-3) protein in synovial tissues and cultured synovial fibroblasts obtained from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and to investigate the consequences of stimulation of cultured synovial fibroblasts with TLR-3 ligands.

Methods. TLR-3 expression in synovial tissues was determined by immunohistochemistry and immunofluorescence, and expression in cultured RA synovial fibroblasts (RASFs) was determined by fluorescence-activated cell sorting and real-time polymerase chain reaction techniques. TLR-3 signaling was assessed by incubating RASFs with poly(I-C), lipopolysaccharide, palmitoyl-3-cysteine-serine-lysine-4, or necrotic synovial fluid cells from RA patients in the presence or absence of hydroxychloroquine or Benzonase. Subsequent determination of interferon-β (IFNβ), CXCL10, CCL5, and interleukin-6 (IL-6) protein production in the culture supernatants was performed by enzyme-linked immunosorbent assays.

Results. TLR-3 protein expression was found to be higher in RA synovial tissues than in OA synovial tissues. TLR-3 expression was localized predominantly in the synovial lining, with a majority of the TLR-3-expressing cells coexpressing fibroblast markers. Stimulation of cultured RASFs with the TLR-3 ligand poly(I-C) resulted in the production of high levels of IFNβ, CXCL10, CCL5, and IL-6 protein. Similarly, coincubation of RASFs with necrotic synovial fluid cells from patients with RA resulted in up-regulation of these cytokines and chemokines in a TLR-3-dependent manner.

Conclusion. Our findings demonstrate the expression of TLR-3 in RA synovial tissue and the activation of RASFs in vitro by the TLR-3 ligand poly(I-C) as well as by necrotic RA synovial fluid cells, and indicate that RNA released from necrotic cells might act as an endogenous TLR-3 ligand for the stimulation of proinflammatory gene expression in RASFs.

Introduction

Recent evidence indicates that the innate immune system plays a decisive role in host defense and self-tolerance (1). Cells of the innate immune system express pattern-recognition receptors, such as the Toll-like receptors (TLRs), which sense certain highly conserved structures that are found on many different bacterial and viral products. The recognition of specific microbial structures, such as lipopolysaccharide (LPS), by TLRs results in the up-regulation of costimulatory molecules in antigen-presenting cells, providing the second signal necessary for generating efficient T cell responses against invading pathogens (2). In contrast, in the absence of a costimulatory TLR signal, T cell receptor stimulation will be followed by a silencing of the T cells. Hence, the innate immune system controls subsequent adaptive immune responses.

Because of this important regulatory role of TLRs, it has been speculated that aberrant TLR signaling may be involved in the generation of autoimmunity. Several animal models of arthritis have been shown to be at least partly dependent on signaling via TLRs (34). Moreover, it has been shown that the injection of bacterial products, such as the TLR-2 ligand peptidoglycan or the TLR-9 ligand CpG DNA, into the joints of mice results in arthritis.
Recently, we demonstrated the expression of TLR-2 in rheumatoid arthritis (RA) synovial tissues (5). We also found that activation of synovial fibroblasts in culture with TLR-2 ligands results in the up-regulation of TLR-2 expression and the production of proinflammatory cytokines (6). Moreover, a variety of chemokines typically found in the synovial fluid of RA patients were found to be secreted by fibroblasts stimulated via TLR-2 (7). While certain TLRs are expressed in joint tissue, it is less clear whether specific TLR ligands are present in the joints of patients with nonseptic arthritis. Peptidoglycans and bacterial DNA derived from gut-colonizing bacteria have been detected in the joints of patients with RA but have also been found in the joints of patients with osteoarthritis (OA) (8). The pathogenetic relevance of these bacterial products, however, remains to be established. More interest has been raised by the demonstration that endogenous ligands, some of which can be found in joints, are able to specifically activate certain TLRs. Recently, activation of TLR-3 by double-stranded RNA (dsRNA) released from necrotic cell lines has been described (9). Injection of dsRNA into mice resulted in a self-limited arthritis, suggesting that TLR-3 signaling may contribute to the pathogenesis of arthritis (10). However, it is not clear whether synovial cells express functional TLR-3.

In the present study, we investigated the possible role of TLR-3 in RA by analyzing TLR-3 expression in synovial tissues and isolated cultured synovial fibroblasts. Our results indicate higher TLR-3 protein expression in RA synovial tissues than in OA synovial tissues. Moreover, we found that RNA released by necrotic synovial fluid cells derived from patients with RA can act as an endogenous ligand for TLR-3 on cultured RA synovial fibroblasts (RASFs). The resulting TLR-3 activation induced the expression of type I IFN as well as the expression of Th1-associated chemokines, such as CXCL10 and CCL5. These results suggest an important role of TLR-3 in the activation of synovial fibroblasts in RA.

Patients and Methods

Patients and tissue preparation. Synovial tissues were obtained from patients with RA and OA who were undergoing synovectomy or joint replacement surgery (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). Synovial tissues were divided into 3 parts and were used to isolate synovial fibroblasts for cell culture, to obtain synovial tissues for immunohistochemistry, and to extract total RNA. All RA patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (11).

Immunohistochemistry and immunofluorescence analyses. Immediately after surgery, synovial tissues were embedded in OCT compound (TissueTek TT 4583; Sakura Finetech, Torrance, CA) and snap-frozen in liquid nitrogen. Embedded synovial tissues were maintained at -80°C until cryosectioned. Seven-micrometer sections were prepared, fixed in acetone, dried, and then rehydrated in phosphate buffered saline (PBS).

Endogenous peroxidase activity was blocked using 0.1% H2O2, and endogenous biotin was blocked using a biotin blocking kit from Vector (Burlingame, CA). To inhibit nonspecific binding, slides were incubated for 1 hour in blocking solution (2% fetal calf serum [FCS] in Tris buffered saline [TBS], pH 7.4). Slides were then incubated for 1 hour with 5 µg/ml of affinity-purified goat anti-human TLR-3 polyclonal antibody (MBL International, Woburn, MA). After the primary antibody reaction, the sections were incubated for 30 minutes with biotinylated rabbit anti-goat IgG (DakoCytomation, Glostrup, Denmark) in TBS with 1% bovine serum albumin, followed by incubation for 30 minutes with horseradish peroxidase (HRP)-conjugated streptavidin complex (Vectastain Elite ABC kit; Vector). HRP-labeled cells were visualized using aminoethylcarbazole chromogen substrate (DakoCytomation).

Nuclei were counterstained with hematoxylin. To identify macrophages, slides were additionally incubated for 1 hour with 3 μg/ml of monoclonal mouse anti-human CD68 (DakoCytomation). To detect fibroblasts, slides were additionally incubated with 3 μg/ml of monoclonal mouse anti-human vimentin (DakoCytomation). Bound mouse primary antibodies were detected using alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG antibody (DakoCytomation). AP-labeled cells were visualized using Fast Blue BB reagent: naphthol-AS-MX phosphate dissolved in N,N-dimethylformamide was mixed immediately before use with Fast Blue BB dissolved in TBS, pH 8.5 (Sigma, Basel, Switzerland) and levamisole solution (DakoCytomation). In control experiments, goat IgG and matched mouse IgG isotype controls were used instead of the primary antibodies. For immunofluorescent double staining, we used 10 μg/ml of fluorescein isothiocyanate-conjugated monoclonal mouse antifibroblast antibody ASO2 (Dianova, Hamburg, Germany). TLR-3 protein was detected using the same primary antibodies as for the immunohistochemical analysis, followed by incubation with 3 μg/ml of phycoerythrin (PE)-conjugated donkey anti-goat antibodies (Jackson ImmunoResearch, Soham, UK). All steps were performed at room temperature.

Isolation and culture of synovial fibroblasts. Immediately after surgery, the synovial tissue was minced and digested with Dispase at 37°C for 60 minutes. After washing, the cells were grown in Dulbecco's minimum essential medium (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 50 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 0.2% Fungizone (all from Gibco Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO2. For the experiments, cultured synovial fibroblasts were used between passages 4 and 8.

Reagents and stimulation assays. Cultured synovial fibroblasts were grown in 12-well culture plates (6 × 104 RASFs/well) and subsequently stimulated with the following agents: poly(I-C) (20 μg/ml; InvivoGen, San Diego, CA), LPS from Escherichia coli (100 ng/ml; List Biological Laboratories, Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) 300 ng/ml (InvivoGen, San Diego, CA) and recombinant interferon-β (IFNβ; PBL Biomedical, Piscataway, NJ).

For stimulation assays with necrotic synovial fluid cells, RASFs were cultured in 6-well plates (8 × 104 RASFs/well). Synovial fluid cells were isolated by centrifugation of fresh synovial fluid aspirates derived from patients with RA. Necrotic synovial fluid cells were

prepared by freeze-thawing synovial fluid cells 3 times. Then, necrotic synovial fluid cells were incubated with or without Benzonase (10 units/106 cells; Novagen, Madison, WI) at 4°C for 12 hours and added to RASFs at a ratio of 10:1, corresponding to 8 × 105 necrotic synovial fluid cells/well. Hydroxychloroquine (HCQ; 2μg/ml) (Sanofi-Synthelabo, Meyrin, Switzerland) was added to the synovial fibroblast cultures 30 minutes prior to stimulation, when indicated. To detect IFNβ messenger RNA (mRNA) expression, isolation of total RNA from synovial fibroblasts was performed after 5 hours of stimulation. Additional cultures were stimulated for 24 hours, and total RNA was isolated from synovial fibroblasts to determine TLR-3 mRNA expression. The culture supernatants were collected and maintained at -80°C for subsequent enzyme-linked immunosorbent assays (ELISAs). All reagents were tested routinely for endotoxin using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Endotoxin levels did not exceed 0.1 endotoxin unit/ml (detection limit) in the tested samples.

ELISAs for Interleukin-6 (IL-6), CCL5, CXCL10, and IFNβ. ELISAs were performed to detect IL-6 protein using an OptEIA kit (BD PharMingen, San Diego, CA) and CCL5 and CXCL10 using DuoSet ELISA development systems (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions. IFNβ protein was detected using a human IFNβ ELISA kit (PBL Biomedical). Absorption was measured at 450 nm, and data were analyzed using Revelation version 4.22 software (Dynex Technologies, Denkendorf, Germany).

Flow cytometry. Cultured synovial fibroblasts were harvested with the use of Accutase (PAA Laboratories, Linz, Austria). For intracellular staining, polyclonal antibody against human TLR-3 and PE-labeled donkey anti-goat IgG were used. Briefly, cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD PharMingen). Permeabilized cells were then incubated for 30 minutes on ice with 5 μg/ml of anti-human TLR-3 antibodies or goat IgG as isotype control. Cells were washed with BD Perm/Wash solution and subsequently incubated on ice for 30 minutes with 3μg/ml of PE-labeled donkey anti-goat IgG. After 2 more washing steps with BD Perm/Wash solution, cells were resuspended in staining buffer (2% FCS/0.1% sodium azide in PBS) and analyzed on a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences, San Diego, CA).

Real-time polymerase chain reaction (PCR). Total RNA from cultured synovial fibroblasts or synovial tissues was isolated with the RNeasy MiniPrep kit (Qiagen, Basel, Switzerland), including treatment with RNase-free DNase. To generate complementary DNA (cDNA), total RNA was reverse transcribed using murine leukemia virus reverse transcriptase (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's protocol. Non-reverse-transcribed samples were used as negative controls. Quantification of specific mRNA was performed by single-reporter real-time PCR using the ABI Prism 7700 Sequence Detection system (Applied Biosystems). The primer pair and probe for quantification of TLR-3 mRNA levels were synthesized by Microsynth (Balgach, Switzerland): 5′-CCT-GGT-TTG-TTA-ATT-GGA-TTA-ACG-A-3′ (forward primer), 5′-TGA-GGT-GGA-GTG-TTG-CAA-AGG-3′ (reverse primer), 5′-ACC-CAT-ACC-AAC-ATC-CCT-GAG-CTG-TCA-A-3′ (probe). IFNβ mRNA levels were quantified using predesigned, gene-specific TaqMan probe and primer sets (TaqMan Gene Expression Assays; Applied Biosystems). The endogenous control 18S cDNA was used to correct the results, according to the comparative threshold cycle (Ct) method for relative quantification, as described by the manufacturer. Differences in Ct values (ΔCt) between the sample and the 18S cDNA were calculated. Relative expression levels were calculated according to the following formula: ΔΔCt = ΔCt (stimulated sample) - ΔCt (unstimulated sample). The value used to plot relative expression was calculated according to the expression $2^{-\Delta\Delta Ct}$.

Statistical analysis. Values are presented as the mean ± SEM. The Mann-Whitney U test and Student's 2-tailed t-test were used where appropriate for statistical evaluation of the data by SPSS software (SPSS, Chicago, IL). P values less than 0.05 were considered significant.

Results

Characterization of TLR-3 expression in synovial tissue from patients with RA and OA. Immunohistochemical analysis was performed to investigate whether TLR-3 protein is expressed in synovial tissues from the joints of patients with RA and OA. TLR-3 protein was found to be broadly expressed in all synovial tissue sections derived from the 7 RA patients. In particular, there was a pronounced expression in the synovial lining (Figures 1A and B). In contrast to RA tissues, TLR-3 protein expression was markedly reduced in synovial tissues derived from 4 OA patients (Figures 1D and E).

The morphology of TLR-3-expressing cells (inset in Figure 1B) and the prominent localization of TLR-3 protein in the synovial lining suggested that synovial fibroblasts could represent the major TLR-3-positive cell population. Therefore, to further characterize the TLR-3-expressing cells, tissue sections were double stained for TLR-3 and vimentin or for TLR-3 and CD68. The majority of synoviocytes expressing TLR-3 stained positive for the fibroblast marker vimentin, indicating that most of the cells in the synovium that expressed TLR-3 were synovial fibroblasts (Figure 1G). Analysis by immunofluorescent double staining with fibroblast-specific ASO2 and TLR-3 antibodies confirmed that TLR-3 protein is expressed by synovial fibroblasts (Figures 1J-L). In the sections analyzed, coexpression of TLR-3 with the macrophage marker CD68 was also identified; however, most of the cells expressing TLR-3 did not express CD68 (Figure 1H).

To verify the differential expression of TLR-3 in RA compared with OA synovial tissues, real-time PCR of total RNA extracts from synovial tissue was performed. TLR-3 mRNA could be detected in all 7 RA samples and all 4 OA samples tested. However, expression of TLR-3 mRNA was significantly higher in the RA synovial tissue (6-fold difference; mean ± SEM ΔCt 15.33 ± 1.79 in RA samples and 17.93 ± 0.81 in OA samples) ($P = 0.024$).

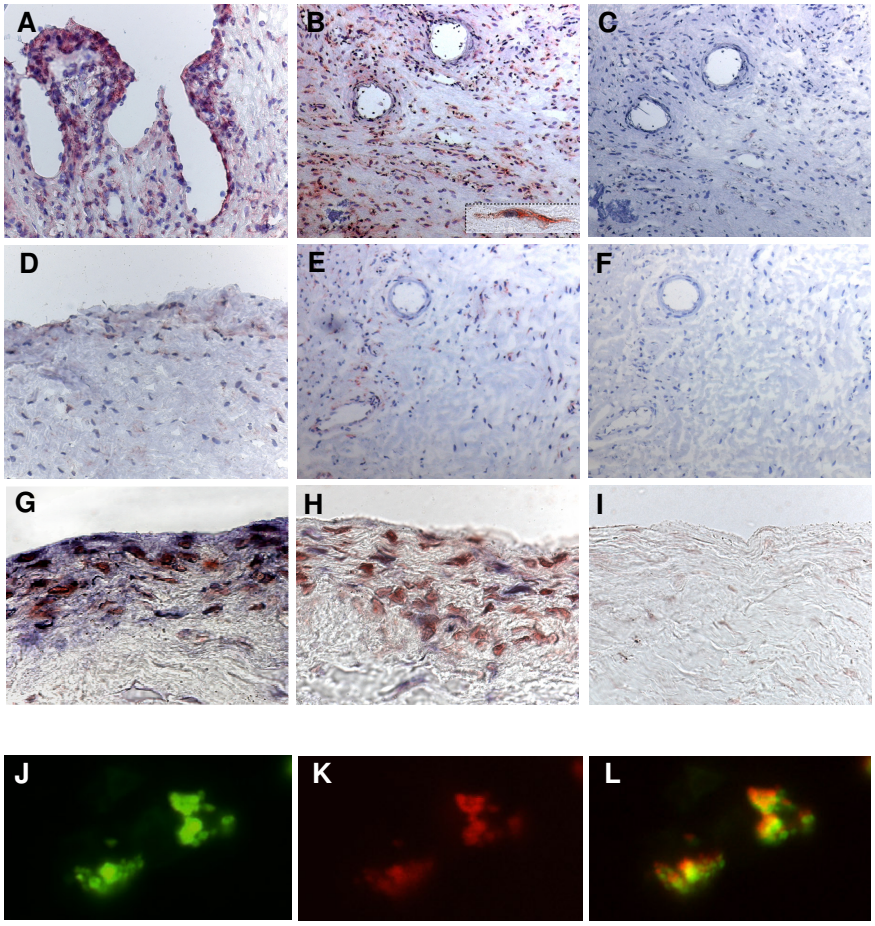

Figure 1. Detection of Toll-like receptor 3 (TLR-3) protein in synovial tissue from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Tissue sections from patients with RA (A-C and G-I) and OA (D-F) were stained with anti-TLR-3 antibodies (A, B, D, and E), with the respective isotype control antibodies (C and F), or with an irrelevant isotype control antibody (I). Shown are both the synovial lining (A and D) and the sublining (B, C, E, and F). Inset, Morphology of TLR-3-expressing cells. TLR-3 protein is shown as red staining. Nuclei were stained with hematoxylin (A-F). Immunohistochemical double stainings were performed with anti-TLR-3 antibodies (red) and antivimentin (blue) (G) as well as with anti-TLR-3 and anti-CD68 (H). Immunofluorescent double staining of sections from RA patients was performed with fibroblast-specific ASO2 antibodies (green) (J) and anti-TLR-3 antibodies (red) (K); an overlay of ASO2-positive and TLR-3-positive cells is also shown (L). (Original magnification × 100 in B, C, E, and F; × 200 in A and D; × 400 in inset, G, H, and I; × 630 in J, K, and L.)

Expression of TLR-3 protein by synovial fibroblasts in vitro. To confirm the results of the immunohistochemical analysis, basal TLR-3 protein expression of cultured synovial fibroblasts was assessed by flow cytometry. A majority of RASFs expressed TLR-3 protein constitutively (mean ± SEM mean fluorescence intensity [MFI] 82.1 ± 8.8; isotype control MFI 32.9 ± 4.9). Moreover, stimulation of RASFs with the TLR-3 agonist poly(I-C) up-regulated the expression of TLR-3 protein by almost 2-fold (MFI 147.0 ± 34.8) as compared with unstimulated cultures (Figure 2A).

To assess the specificity of TLR-3 up-regulation, RASFs and OASFs were stimulated with the TLR-3 ligand poly(I-C), with the TLR-4 ligand LPS, or with the TLR-2 ligand Pam3CSK4 (Figure 2B). TLR-3 mRNA expression was analyzed by real-time PCR 24 hours following stimulation. Poly(I-C) up-regulated TLR-3 mRNA in RASFs a mean ± SEM of 14.2 ± 2.0-fold and in OASFs a mean ± SEM of 9.3 ± 2.4-fold compared with unstimulated cultures, with a statistically significant difference between RASFs and OASFs. LPS stimulation induced a small up-regulation of TLR-3 mRNA (3.0 ± 1.0-fold in RASFs and 2.2 ± 0.8-fold in OASFs), but the difference was not statistically significant. Pam3CSK4 did not show any TLR-3 mRNA up-regulation in either case. These results demonstrate the constitutive expression of TLR-3 in synovial fibroblasts and a preferential up-regulation of TLR-3 in synovial fibroblasts from patients with RA in the presence of the TLR-3 agonist poly(I-C).

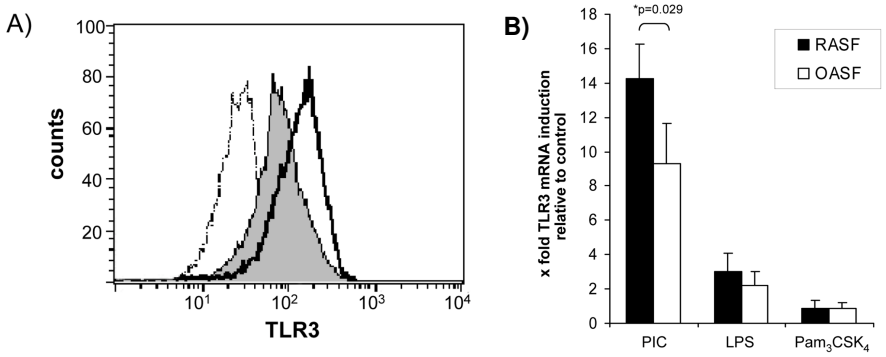

Figure 2. Expression of Toll-like receptor 3 (TLR-3) in cultured synovial fibroblasts. A, Rheumatoid arthritis synovial fibroblasts (RASFs) were cultured for 36 hours with or without 20 µg/ml of poly(I-C). TLR-3 protein expression was analyzed by flow cytometry in unstimulated RASFs (shaded histogram) and poly(I-C)-stimulated RASFs (histogram with thick line), as compared with control IgG (histogram with thin line). A total of 10,000 cells were analyzed per sample. Shown is a representative histogram from 4 different experiments. B, Cultures of RASFs and osteoarthritis synovial fibroblasts (OASFs) (n = 4 patients per group) were stimulated for 24 hours with the indicated TLR ligands or were left untreated. Levels of TLR-3 expression were determined by real-time polymerase chain reaction. Values are the mean and SEM up-regulation of TLR-3 expression as compared with unstimulated cultures. The mean difference in the comparative threshold cycle (ΔCt) value for TLR-3 in untreated cultures was 14.22 for RASFs and 15.27 for OASFs. PIC = poly(I-C); LPS = lipopolysaccharide; Pam3CSK4 = palmitoyl-3-cysteine-serine-lysine-4.

Induction of IFNβ, CXCL10, CCL5, and IL-6 in RASFs following stimulation with poly(I-C). In contrast to TLR-2 and other known TLR family members, TLR-3 signals exclusively through a myeloid differentiation factor 88 (MyD88)-independent pathway using the adaptor molecule TRIF, whereas TLR-4 uses both the MyD88-dependent and the MyD88-independent pathways, with TRIF and TRAM as adaptors. In addition to NF-κB and MAP kinases, the TRIF-dependent pathways activate the transcription factor interferon regulatory factor 3 (IRF-3), leading to the induction of type I IFN expression. To assess the TRIF-

dependent pathways in cultured RASFs, cells were stimulated with poly(I-C), LPS, and Pam3CSK4. After 24 hours of stimulation, the supernatants were tested by ELISA for the presence of TRIF-dependent and TRIF-independent cytokines and chemokines.

IFNβ in RASFs was highly induced by poly(I-C) and was induced to a lesser extent by LPS, whereas Pam3CSK4 had no effect (Figure 3). Moreover, the Th1-associated chemokines CXCL10 and CCL5 were most strongly induced after stimulation with poly(I-C). In response to LPS, RASFs produced significant amounts of CXCL10 and CCL5 protein, whereas Pam3CSK4 induced only CCL5. High amounts of the proinflammatory cytokine IL-6 were detected in supernatants from RASF cultures stimulated with each of the 3 TLR ligands; however, poly(I-C) was the most effective stimulator (Figure 3).

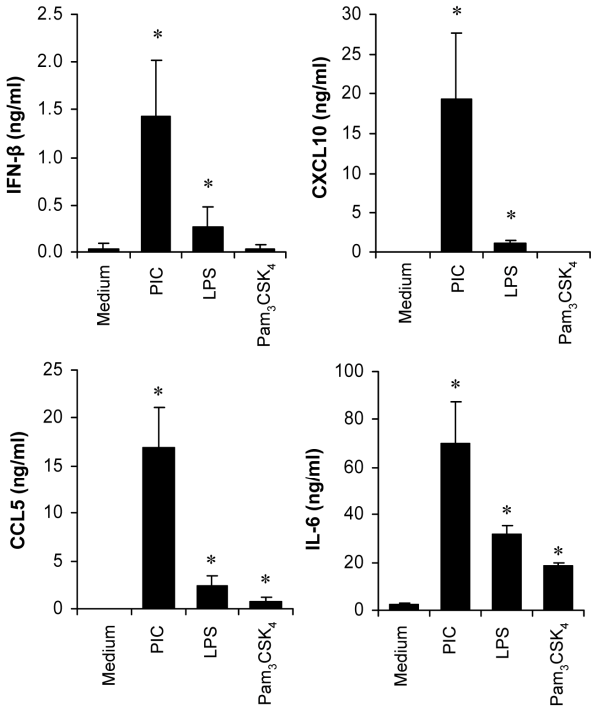

Figure 3. Production of interferon-β (IFNβ), CXCL10, CCL5, and interleukin-6 (IL-6) protein by rheumatoid arthritis synovial fibroblasts (RASFs) following stimulation with poly(I-C) (PIC). RASF cultures were stimulated with the indicated Toll-like receptor ligands or were left untreated. Concentrations of IFNβ, CXCL10, CCL5, and IL-6 in the culture supernatants were determined after 24 hours with enzyme-linked immunosorbent assays. Values are the mean and SEM of 4-6 different RASF cultures. *= *P* < 0.02 versus untreated cultures. LPS = lipopolysaccharide; Pam_3CSK_4 = palmitoyl-3-cysteine-serine-lysine-4

Induction of CXCL10 expression by IFNβ released by poly(I-C) stimulated RASFs. Recent studies suggest that type I IFNs are able to induce CXCL10 gene expression (12). We

therefore analyzed whether IFNβ regulates the chemokine gene expression in poly(I-C)-stimulated RASFs. RASFs were stimulated for 24 hours with poly(I-C) in the presence of neutralizing anti-IFNβ antibodies or rabbit IgG as isotype control, and the production of CXCL10, CCL5, and IL-6 protein by RASFs was measured in the supernatants by ELISA (Figure 4A). Whereas the CXCL10 concentrations were significantly reduced when neutralizing anti-IFNβ antibodies were added, no significant effect was detected for the expression of CCL5 or IL-6. These data suggest that CXCL10 gene expression in response to TLR-3 activation is at least partly dependent on IFNβ. We therefore analyzed whether exogenous IFNβ was able to stimulate CXCL10 production in RASFs. RASFs were incubated with various concentrations of IFNβ for 24 hours, and CXCL10 protein production was subsequently analyzed by ELISA. Consistent with the findings shown in Figure 4A, exogenous IFNβ induced CXCL10 protein secretion in a dose-dependent manner (Figure 4B).

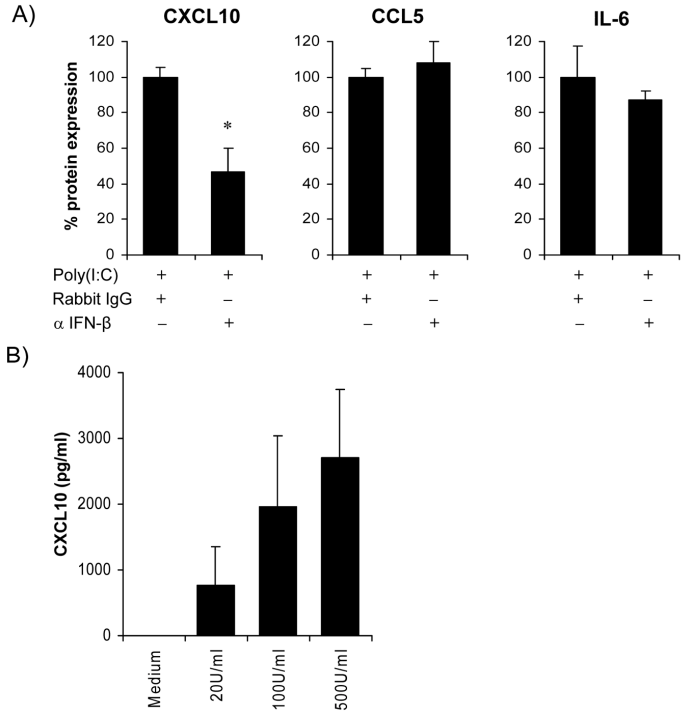

Figure 4. Regulation of CXCL10 gene expression by interferon-β (IFNβ). A, Cultured rheumatoid arthritis synovial fibroblasts (RASFs) were incubated with 20 μg/ml of poly(I-C) in the presence of 10 μg/ml of IFNβ neutralizing antibodies (α IFNβ) or 10 μg/ml of rabbit IgG as a control, and levels of CXCL10, CCL5, and interleukin-6 (IL-6) were determined. *= *P* < 0.02 versus reference cultures. B, Levels of CXCL10 in RASF supernatants treated with various concentrations of recombinant IFNβ. Culture supernatants were collected after 24 hours of stimulation and analyzed by enzyme-linked immunosorbent assay. Values are the mean and SEM of 3 individual RASF cultures.

Inhibition of TLR-3 signaling in synovial fibroblasts by HCQ. HCQ inhibits endosomal acidification, on which signaling of the intracellularly located TLRs, such as TLRs 3, 7, 8,

and 9, depend. Since synovial fibroblasts do not express TLRs 7, 8, and 9, we used HCQ as a specific TLR-3 inhibitor in cultures of RASFs stimulated with poly(I-C), LPS, or Pam3CSK4. The induction of IFNβ, CXCL10, CCL5, and IL-6 in response to poly(I-C) was almost completely abolished when RASFs were pretreated with 2 μg/ml of HCQ (Table 1). In contrast, HCQ did not have such an effect on the cytokine and chemokine production by RASFs in response to LPS or Pam3CSK4.

Table I: Specific inhibition of TLR-3 in RASFs

	% IFN-β	% CXCL10	% CCL5	% IL-6
poly(I-C)	100	100	100	100
poly(I-C) + HCQ	5.0 ± 2.8	0.7 ± 0.6	0.7 ± 0.5	4.3 ± 1.6
LPS	100	100	100	100
LPS +HCQ	109.2 ± 45.5	101.2 ± 22.7	102.7 ± 23.1	110.5 ± 17.4
Pam_3CSK_4	ND	ND	100	100
Pam_3CSK_4 + HCQ	ND	ND	91.4 ± 5.3	112.7 ± 26.8

[*] **Rheumatoid arthritis synovial fibroblasts (RASFs) were stimulated for 24 hours with the indicated Toll-like receptor (TLR) ligands in the presence or absence of 2 μg/ml of hydroxychloroquine (HCQ). Culture supernatants were collected, and protein levels were determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM of at least 3 individual experiments. IFNβ = interferon-β; IL-6 = interleukin-6; LPS = lipopolysaccharide; Pam_3CSK_4 = palmitoyl-3-cysteine-serine-lysine-4; ND = not done.**

Necrotic synovial fluid cell stimulation of RASFs via TLR-3. It has been shown that mRNA released from necrotic cells can act as an endogenous ligand for TLR-3 in dendritic cells (9). Since necrotic cells can be detected in the synovial fluid of patients with RA, we examined the stimulatory effects of necrotic synovial fluid cells from RA patients on cultured RASFs. RASFs cultured in the presence of necrotic synovial fluid cells up-regulated the expression of IFNβ mRNA and the production of IL-6, CXCL10, and CCL5 protein (Figure 5A). The stimulatory effect was dependent on the concentration of necrotic cells added to the cultures (Figure 5B). The addition of HCQ resulted in a significant reduction of the stimulatory effect of necrotic synovial fluid cells, suggesting TLR-3 dependency.

To examine whether RNA is the effector molecule for TLR-3 activation, necrotic synovial fluid cells were incubated with Benzonase for 12 hours at 4°C prior to stimulation. Benzonase is an endonuclease that degrades all forms of RNA into oligomers of 2-5 nucleotides in

length. RNA digestion from necrotic synovial fluid cells by Benzonase significantly decreased the expression of IFNβ mRNA as well as the protein concentrations of the tested chemokines and cytokines in the RASF cultures (Figure 5A). These results suggest that RNA derived from necrotic synovial fluid cells stimulates synovial fibroblasts via TLR-3.

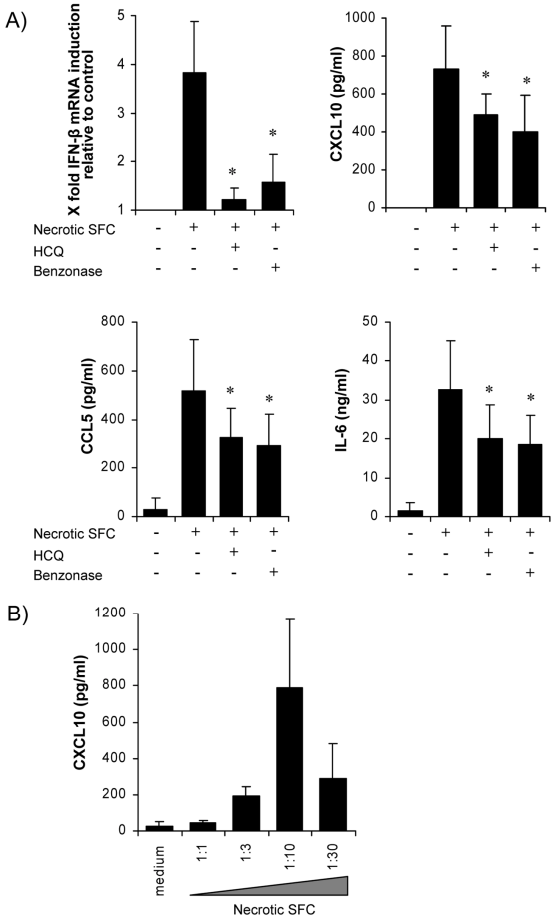

Figure 5. Production of interferon-β (IFNβ), CXCL10, CCL5, and interleukin-6 (IL-6) by rheumatoid arthritis synovial fibroblasts (RASFs) following stimulation with necrotic synovial fluid cells (SFC). A, Cultured RASFs were stimulated with necrotic synovial fluid cells in the presence or absence of 2 μg/ml of hydroxychloroquine (HCQ). Necrotic synovial fluid cells were pretreated with Benzonase (as indicated). After 5 hours of stimulation, total RNA was extracted from RASFs, and IFNβ mRNA induction was analyzed by real-time polymerase chain reaction. Additional cultures were stimulated for 24 hours, and CXCL10, CCL5, and IL-6 protein production was determined in the supernatants by enzyme-linked immunosorbent assay (ELISA). B, Cultured RASFs were stimulated with increasing numbers of necrotic cells, as indicated by the RASF/necrotic cell ratio, and CXCL10 in the supernatants was measured by ELISA. Values are the mean and SEM of at least 5 different RASF cultures. *= *P* < 0.04 versus RASFs stimulated with necrotic synovial fluid cells in the absence of inhibitors. Necrotic synovial fluid cells cultured without RASFs did not produce any of the cytokines or chemokines assayed (data not shown).

Discussion

Activation of the innate immune system via Toll-like receptors leads to the induction of the expression of proinflammatory cytokines and chemokines. Both play an important role in the development of the joint inflammation characteristic of RA. We have previously demonstrated the expression of functional TLR-2 in synovial fibroblasts from patients with RA (56). The finding that TLR-2 expression was increased in RA synovial tissue as compared with OA synovial tissue suggested that TLR signaling is active in RA. Further evidence of a role of TLRs in arthritis is provided by studies of animal models of arthritis. Streptococcal cell wall-induced arthritis was shown to be dependent on TLR-2 signaling (4). Moreover, it was shown that LPS can circumvent the need for IL-1 for development of antibody-transfer arthritis in the K/BxN model, demonstrating a role of TLR ligands in later stages of the pathogenesis of arthritis (3). Although microbial products such as bacterial DNA and peptidoglycans have been detected in the joints of patients with RA, their pathogenic significance is unclear.

The recent identification of endogenous ligands for several different TLRs has generated great interest because of their potential importance for autoimmunity. For example, heat-shock proteins 60 and 70 have been shown to be ligands of TLR-2 and TLR-4 and have previously been implicated in the pathogenesis of RA (13-16). Fibrinogen is another TLR-4 ligand that is present in joints (17). Moreover, it has been demonstrated that chromatin-containing immune complexes may give rise to the production of rheumatoid factor autoantibodies by the synergistic engagement of B cell receptor and TLR-9 (18). Necrotic cells have been demonstrated to activate macrophages in a TLR-2-dependent manner, although the nature of the ligand remains obscure (19). Viral dsRNA is a ligand for TLR-3, whereas the single-stranded form activates TLR-8 (20, 21). Kariko et al (9) have demonstrated that stimulation of TLR-3 is not restricted to viral RNA, but also results from incubation with in vitro-transcribed mRNA and mRNA released from necrotic cells. These results have established that dsRNA sequences contained in mRNA may serve as an endogenous TLR-3 ligand.

In this study, we analyzed TLR-3 expression in synovial tissues and its functional aspects in vitro. TLR-3 was found to be expressed in all samples derived from patients with RA and OA; however, staining of OA sections was weak compared with the staining of RA sections. Stimulation of cultured RASFs with poly(I-C) resulted in increased TLR-3 expression. These

findings are compatible with active TLR-3 signaling in RA, although it remains unclear whether increased TLR expression represents a secondary phenomenon related to the inflammatory reaction in the joints. We therefore analyzed the consequences of TLR-3 stimulation in synovial fibroblasts in vitro.

Stimulation with the TLR-3 ligand poly(I-C) resulted in the up-regulation of the proinflammatory cytokine IL-6 and the chemokine CCL5. This effect of poly(I-C) is similar to the expression profile seen with TLR-2 stimulation (6) and is consistent with the activation of NF-κB, which has been shown to occur upon TLR-3 stimulation in a MyD88-independent manner via tumor necrosis factor receptor-associated factor 6 (22). In contrast to all other TLRs (except TLR-4), TLR-3 signals via a MyD88-independent pathway using the adaptor molecule TRIF, which leads not only to NF-κB activation, but also to the activation of IRF-3, inducing the expression of type I IFN and IFN-responsive genes. Correspondingly, poly(I-C) stimulation of RASFs induced the expression of IFNβ and the chemokine CXCL10. The up-regulation of CXCL10 by poly(I-C) seen in RASFs was at least partly dependent on IFNβ production, since it could be inhibited by anti-IFNβ antibodies. The specificity of the effect of poly(I-C) was confirmed by using HCQ. HCQ blocks endosomal acidification and thereby inhibits signaling of intracellularly located TLRs, such as TLRs 3, 7, 8 and 9. Since TLRs 7, 8, and 9 are not expressed in RASFs (ref. 6 and Brentano F, et al: unpublished observations), HCQ acts as a specific TLR-3 blocker.

Both IFNβ and CXCL10 have previously been described as being up-regulated in RA (23-26). CXCL10 is a chemokine that attracts activated T cells and natural killer cells. Expression of CXCR3, the receptor for CXCL10, has been associated with a Th1 phenotype (27). Whereas IFNγ is the main inducer for CXCL10 expression in peripheral blood mononuclear cells, dsRNA has a stronger inducing effect in fibroblasts, acting synergistically with IFNγ (28). In addition, up-regulation of CXCL10 secretion was demonstrated in cocultures of fibroblast-like synoviocytes and leukocytes, and was dependent on cell contact but independent of cytokines (23). Interestingly, when combinations of cytokines and bacterial compounds were used to stimulate fibroblasts, very high levels of CXCL10 (up to the range of μg/million cells) were observed (28). In our experiments, poly(I-C) stimulation reached concentrations up to 500 ng/million cells. Such high concentrations might significantly act to direct the adaptive immune response toward a Th1 response. This concept is supported by recent data showing

the suppression of diabetes in the NOD mouse model, a Th1-dominated disease, by CXCL10 neutralization (29).

Type I IFNs have pleiotropic effects on the cells of the immune system. They induce activation of immature dendritic cells, promote B and T cell maturation, keep activated CD8+ and CD4+ cells alive, and promote macrophage maturation and inducible nitric oxide synthase production. In contrast, they also have antiproliferative and proapoptotic effects on T cells (for review, see ref. 30). With regard to IFNβ, available data from mouse models of arthritis suggest a beneficial effect of continuous administration of this cytokine. IFNβ treatment in mice as well as primates with collagen-induced arthritis inhibited the development of arthritis (31, 32). A recent randomized controlled clinical trial, however, reported no benefit of daily administration of IFNβ (33). In vitro studies suggest beneficial as well as detrimental effects of IFNβ. Whereas IFNβ was shown to be involved in stromal cell rescue of T cell blasts (26), possibly resulting in inappropriate T cell survival, another study demonstrated induction of IL-1 receptor antagonist (34, 35). In our experiments, however, there was no indication of an inhibitory effect of IFNβ on the expression of proinflammatory cytokines and chemokines by RASFs stimulated via TLR-3.

TLR pathways are activated during infections, contributing to an efficient host defense, but are self limited with the clearing of the microorganism. Uncontrolled TLR signaling could theoretically lead to autoimmune disease; however, so far, no specific disease has been linked to a defect in TLR regulation. In addition to up-regulated TLR expression, the availability of TLR ligands may be responsible for the enhanced activity of the signaling pathways.

Necrotic cells may be found in RA joints as a result of inflammatory and destructive processes. Whereas necrotic cells can easily be detected in synovial fluid (e.g., with propidium iodide staining, we consistently found at least 10% necrotic cells in synovial fluid from RA patients), necrotic cells are usually not present in large amounts in the synovial tissue. However, apoptotic cells in synovial tissue may undergo secondary necrosis (36).

In an attempt to assess the effects of necrotic cells on synovial fibroblasts under conditions as close as possible to those found in vivo in the arthritic joint, we used necrotic synovial fluid cells from RA patients to stimulate cultured RASFs. Interestingly, the necrotic synovial fluid cells efficiently up-regulated proinflammatory cytokines and chemokines, with a profile

resembling that of RASFs stimulated with poly(I-C). The induction of IL-6, CCL5, and CXCL10 protein and IFNβ mRNA was significantly reduced by the addition of HCQ, which inhibited TLR-3 signaling, but not TLR-2 and TLR-4 signaling. Similarly, the RNA-degrading enzyme Benzonase significantly decreased the response of RASFs to the necrotic synovial fluid cells. These results indicate that mRNA containing short dsRNA sequences that are released from necrotic synovial fluid cells are sufficient to activate cultured human RASFs, resulting in the induction of proinflammatory gene expression. These findings extend those of a recent study that demonstrated the activation of human endometrial cells with U1 RNA containing dsRNA repeats (37). The U1 RNA was derived from U1 RNP, autoantibodies to which can be found in a subset of patients with collagen vascular disease.

Cell-free synovial fluid from patients with RA also stimulated RASFs. However, in this case, the presence of cytokines such as tumor necrosis factor α, IL-1β, or IFNβ may be responsible for the stimulatory effect. This is suggested by the fact that the activation induced by the cell-free synovial fluid could not be blocked by HCQ (data not shown).

In summary, we have demonstrated the expression of TLR-3 in a majority of synovial fibroblasts from the joints of patients with RA and the activation of RASFs in vitro by dsRNA of synthetic or endogenous origin. Based on our findings, we propose that, in addition to stimulatory effects of cytokines, local tissue-destructive events, whether caused by an infection involving the joint or by noninfectious processes, may lead to the release of endogenous dsRNA, activating tissue-resident synovial fibroblasts via TLR-3. In susceptible individuals, increased TLR-3 expression and expression of tissue-destructive enzymes by RASFs might then contribute to the perpetuation of the disease.

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CHAPTER 4

Pre-B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities

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Abstract

Objective. To study possible mechanisms that mediate induction of the recently described adipocytokine pre-B cell colony-enhancing factor (PBEF) in joints of patients with rheumatoid arthritis (RA), and to analyze whether levels of PBEF correlate with disease severity and whether PBEF itself has the potential to act as a proinflammatory and destructive mediator in RA.

Methods. RA synovial fibroblasts (RASFs) and monocytes were stimulated with Toll-like receptor (TLR) ligands, cytokines, and recombinant human PBEF or were transfected with PBEF expression constructs or with PBEF-specific small interfering RNA. Production of interleukin-6 (IL-6), IL-8, and tumor necrosis factor alpha (TNFα) was measured by enzyme-linked immunosorbent assay, and expression of matrix metalloproteinases (MMPs) was assessed by real-time polymerase chain reaction. PBEF expression in synovial tissue, synovial fluid, serum, and SFs was assessed by immunohistochemistry, in situ hybridization, Western blotting, and enzyme immunoassays.

Results. In RASFs, PBEF was up-regulated by TLR ligands and cytokines that are characteristically present in the joints of patients with RA. In synovial tissue, RASFs were the major PBEF-expressing cells. A predominance of PBEF was found in the synovial lining layer and at sites of invasion into cartilage. Levels of PBEF in serum and synovial fluid correlated with the degree of inflammation and clinical disease activity. Moreover, PBEF itself activated the transcription factors NF-kB and activator protein 1 and induced IL-6, IL-8, MMP-1, and MMP-3 in RASFs as well as IL-6 and TNFalpha in monocytes. PBEF knockdown in RASFs significantly inhibited basal and TLR ligand-induced production of IL-6, IL-8, MMP-1, and MMP-3.

Conclusion. Our findings establish PBEF as a proinflammatory and destructive mediator of joint inflammation in RA and identify PBEF as a potential therapeutic target.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that ultimately leads to the progressive destruction of joint cartilage and bone. Synovial fibroblasts (SF) and inflammatory cells such as macrophages and T cells play key roles in this process. There is mounting evidence for an important function of innate immunity in the pathogenesis of RA (1). Activation of cells by microbial components and also by endogenous molecules via pattern recognition receptors, such as Toll-like receptors (TLR), results in the production of a variety of cytokines, chemokines and matrix metalloproteinases (MMPs) some of which can characteristically be found in RA (2). We have previously reported the induction of the proinflammatory cytokine IL-6 and the chemokines IL-8, GCP-2, MCP-2 and RANTES by the TLR2 ligand bacterial peptidoglycan in SF (3, 4). In a subsequent study we have demonstrated overexpression of TLR3 in RA synovial tissue and established that RNA released by necrotic synovial fluid cells can act as endogenous ligand for TLR3 on cultured RA synovial fibroblasts (RASF) (5). For the current study we performed subtractive hybridisation experiments with untreated and poly(I-C) treated RASF to investigate novel TLR3 dependent gene regulation. We found pre-B cell colony enhancing factor (PBEF) to be upregulated by the TLR3 ligand poly(I-C).

PBEF was originally cloned from a cDNA library of activated human peripheral blood mononuclear cells (PBMCs) and identified as a secreted protein that enhances the effect of stem cell factor and IL-7 on pre-B-cell colony formation (6). Later it has become evident that PBEF is a multifunctional protein by having nicotinamide phosphoribosyltransferase, adipokine and cytokine activities. In smooth muscle cells (SMC) intracellularly located PBEF regulates NAD+-dependent reactions and promotes the acquisition of a mature SMC phenotype (7, 8). Fukuhara et al. has reported the expression of PBEF in visceral fat adipocytes and that its insulin-like effect is dependent on the binding to the insulin-receptor (9). Because of its presence in the visceral fat, PBEF is also referred to as Visfatin. Furthermore antiapoptotic effects of PBEF are documented in neutrophils (10). Regarding cytokine activities, PBEF induces IL-6 and IL-8 in amniotic cells (11), whereas downregulation of PBEF results in the inhibition of thrombin-stimulated increase of IL-8 secretion in pulmonary artery endothelial cells (12).

Levels of PBEF in serum and synovial fluids are elevated in RA patients (13, 14). Furthermore Nowell et al. demonstrated increased synovial expression of PBEF in antigen induced arthritis in mice. The upregulation of PBEF was shown to be regulated by IL-6 trans-signalling via STAT-3. However, the role of PBEF in joint inflammation remains to be determined.

We investigated whether PBEF is involved in the inflammatory and destructive processes in the rheumatoid joint. The expression of PBEF was examined in synovial tissues, sera and synovial fluids obtained from patients with RA. We show that levels of PBEF correlate with the degree of inflammation and clinical disease activity in RA patients. Stimulation of RASF and primary monocytes with rhPBEF as well as PBEF overexpression and knockdown experiments revealed that PBEF acts as a proinflammatory mediator by triggering the release of cytokines, chemokines and destructive enzymes, characteristically found in the inflamed joints of RA patients. Thus, our findings indicate that PBEF is a marker of inflammation and that PBEF itself promotes inflammatory and destructive processes in joints of RA patients.

Patients and Methods

Patients and tissue preparation. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and patients with osteoarthritis (OA), after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASFs and OASFs were isolated from synovial tissue, digested by collagenase, and used after passages 4-8, as previously described (5). To obtain tissue sections, synovial specimens were fixed in paraformaldehyde and embedded in paraffin. Sera and synovial fluid from patients with RA and patients with OA were collected, centrifuged, and stored at -80°C until analyzed. Before analysis, synovial fluid samples were pretreated for 1 hour at 37°C with 1 mg/ml of hyaluronidase (Fluka, Buchs, Switzerland). All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (15).

Stimulation assays. RASFs and OASFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) and stimulated with the following agents: poly(I-C) (20 µg/ml; Invitrogen, San Diego, CA), lipopolysaccharide (LPS) from Escherichia coli (100 ng/ml; List Biological Laboratories,

Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (bacterial lipoprotein [bLP]) (300 ng/ml; Invitrogen), IL-1β (1 ng/ml; R&D Systems, Abington, UK,) and TNFα (10 ng/ml; R&D Systems). PBMCs were isolated by standard Ficoll density-gradient centrifugation from blood samples from healthy donors. CD14+ monocytes were separated using CD14 MACS MicroBeads according to the manufacturer's protocol (Miltenyi Biotec, Gladbach, Germany). The purity of the CD14+ cell fraction, as assessed by flow cytometry, was consistently >90%. CD14+ cells were cultured in RPMI 1640 supplemented with 5% FCS. RASFs and CD14+ cells were stimulated with rHuPBEF (Phoenix Pharmaceuticals, Belmont, CA) in the presence of polymyxin B sulfate (5 μg /ml) (Sigma, Basel, Switzerland).

Real-time polymerase chain reaction (PCR). Quantification of specific PBEF and MMP messenger RNA (mRNA) was performed by SYBR Green and TaqMan real-time PCR, respectively. The primer sequences used are as follows: for PBEF, forward 5'-AAT ACC CAC CCA ACA CAA GC-3', reverse 5'-TCA CGG CAT TCA AAG TAG GA-3'; for MMP-1, forward 5'-TGT GGA CCA TGC CAT TGA GA-3', reverse 5'-TCT GCT TGA CCC TCA GAG ACC-3', probe 5'-AGC CTT CAA ACT CTG GAG TAA TGT CAC ACC-3'; for MMP-3, forward 5'-GGG CCA TCA GAG GAA ATG AG-3', reverse 5'-CAC GGT TGG AGG GAA ACC TA-3', probe 5'-AGC TGG ATA CCC AAG AGG CAT CCA CAC-3'. The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle method for relative quantification, as described by the manufacturer.

In situ hybridization. PBEF sense and PBEF antisense probes for in situ hybridization were prepared according to methods previously described (16). In situ hybridization was performed using the method described by Kriegsmann et al (17).

Immunohistochemical analysis. Synovial tissue sections were deparaffinized and pretreated with trypsin (1 mg/ml; Sigma). After blocking endogenous peroxidase and nonspecific binding, slides were incubated overnight at 4°C with anti-human PBEF antibodies (5 μg/ml; Bethyl Laboratories, Montgomery, TX). Sections were then incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, Soham, UK) in Tris buffered saline with 3% bovine serum albumin for 30 minutes at room temperature, followed by incubation for 30 minutes with horseradish peroxidase (HRP)-conjugated streptavidin complex at room temperature (ABC kit; Vector, Peterborough, UK). HRP-labeled cells were visualized using aminoethylcarbazole substrate-chromogen (Dako, Glostrup, Denmark). Nuclei were

counterstained with hematoxylin. To identify subsets of synovial cells expressing PBEF, slides were additionally incubated with monoclonal mouse anti-human CD68 or anti-human vimentin antibodies (2 μg/ml; Dako), respectively. Bound mouse primary antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch). Alkaline phosphatase-labeled cells were visualized using Fast Blue B reagent. In control experiments, rabbit IgG and isotype-matched mouse IgG were used instead of the primary antibodies.

Western blotting. Protein preparation from supernatants: Cultured SFs were grown in T75 culture flasks (7×10^5 cells/flask) in DMEM supplemented with 0.5% FCS and subsequently were stimulated for 24 hours with poly(I-C) or were left untreated. Supernatants were collected and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA). Protein preparation from tissue: Protein was extracted by resuspending the crushed snap-frozen tissue in extraction buffer, with subsequent aceton precipitation overnight at -20°C. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and blotted on Protran nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). Membranes were probed with anti-PBEF antibodies (0.5 μg/ml; Bethyl Laboratories) and detected with HRP-conjugated secondary antibodies, using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were stripped and reprobed with monoclonal mouse anti-human α-tubulin antibodies (Sigma) to confirm similar loading of the gels.

Construction of PBEF plasmids. Total RNA from RASFs was extracted, and 1 μg RNA was transcribed to first-strand cDNA using the Moloney murine leukemia virus reverse transcriptase system (Invitrogen). The cDNA was amplified with the upstream primer, 5'-GCGGGATCCGCGATGATGTGCTGCTTCCAGTTC-3', containing a Bam H1 restriction site and a Kozak sequence for eukaryotic translation. The downstream primer was 5'-CCGCTCGAGCGGCCCGAGATGAATCCTGCG-3', containing an Xho I restriction site. Full-length PBEF fragments, including the signal sequence and fragments encoding the mature PBEF peptide sequence, were cloned into pcDNA3.1/myc-His vector. The recombinant plasmids were transfected into DH5α-competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and sequencing.

Transfection of PBEF small interfering RNA (siRNA) into RASFs.

PBEF stealth siRNAs were designed based on the human PBEF cDNA reference sequence (NM_005746.1), using the BLOCK-iT RNAi Designer (Invitrogen). Stealth106 siRNA PBEF 5'-AAU AAA CUU UGC UUG UGU UGG GUG G-3' and stealth 106 scrambled PBEF 5'-CCA CAA CAA CAA ACG UUG AUC CAU U-3' were used. One day before transfection, RASFs were plated in DMEM/10% FCS without antibiotics, in a 24-well plate (30,000 RASFs per well). For each transfection, 50 nM PBEF stealth siRNA was diluted in 50 µl Opti-MEM I (Invitrogen) without serum and mixed with 1 µl Lipofectamine 2000 in 50 µl Opti-MEM I. After incubation for 20 minutes at room temperature, PBEF stealth siRNA-Lipofectamine 2000 complexes were added to each well. Transfected cells were further incubated at 37°C for 48 hours before culture medium was replaced and RASFs were used for further experiments.

Enzyme-linked immunoabsorbant assay (ELISA) and enzyme immunoassay (EIA). IL-6 and TNFα proteins were detected by ELISA with the OptEIA Kit (BD PharMingen, San Diego, CA), and PBEF/visfatin protein was detected using a human EIA kit (Phoenix Pharmaceuticals), according to the manufacturer's instructions. Absorption was measured at 450 nm, and data were analyzed using Revelation version 4.22 software (Dynex Technologies, Denkendorf, Germany).

Electrophoretic mobility shift assay (EMSA). For EMSA, RASFs were cultured to 80-90% confluency in culture flasks (75 cm^2) and incubated with 100 ng/ml of rHuPBEF. Cells were collected by scratching in ice-cold phosphate buffered saline, at different time points (0, 10, 30, and 90 minutes after stimulation). DNA-binding proteins were extracted from RASFs according to the method described by Andrews and Faller, which utilizes hypotonic lysis followed by high-salt extraction of nuclei (18). The binding EMSA was carried out using a Panomics EMSA Gel Shift kit, according to the manufacturer's instructions (Panomics, Redwood City, CA).

Statistical analysis. The Mann-Whitney U test, Wilcoxon's test, and the nonparametric Spearman's correlation were used, as appropriate, for statistical evaluation of the data by SPSS software (SPSS, Chicago, IL). p-values less than 0.05 were considered significant.

Results

Induction of PBEF in synovial fibroblasts by TLR ligands and inflammatory cytokines.
In order to investigate novel TLR-3-dependent gene regulation, we performed subtractive hybridization between cDNA prepared from poly(I-C)-stimulated and unstimulated RASFs. PBEF was one of the transcripts shown to be up-regulated by TLR-3 activation. The induction of PBEF mRNA in RASFs was validated at various time points after poly(I-C) treatment, by real-time PCR (Figure 1A). The expression of PBEF was significantly up-regulated after 5, 10, and 24 hours of poly(I-C) stimulation, with a peak at 10 hours after stimulation. To investigate the regulation of PBEF expression by other TLR ligands and proinflammatory cytokines present in RA synovial fluid, SFs were additionally treated with the TLR-2 ligand bLP, the TLR-4 ligand LPS, IL-1β, TNFα at optimal concentrations, and PBEF itself. IL-1β potently up-regulated PBEF mRNA, to levels similar to those reached with poly(I-C) stimulation. The stimulatory effects of TNFα, LPS, bLP, and PBEF on the expression of PBEF mRNA were less prominent but were significant 10 hours following stimulation.

To analyze the expression of PBEF at the protein level, cell lysates from RASFs and OASFs treated with the indicated stimuli were subjected to Western blot analysis (Figure 1B). PBEF was found to be constitutively expressed by SFs, and its expression was further increased following stimulation with bLP, poly(I-C), LPS, TNF, IL-1β, or rHuPBEF. Additionally, we observed a tendency toward higher PBEF levels in RASFs compared with OASFs.
In previous studies, PBEF was shown to be a secreted protein, despite the lack of the typical signal peptide that is common to other secreted proteins (10). To analyze whether SFs have the potential to secrete PBEF protein as a cytokine, we performed Western blot analyses for PBEF using supernatants of unstimulated and poly(I-C)-stimulated RASFs and OASFs. Cultured RASFs released PBEF protein constitutively, as shown in the supernatants of unstimulated RASFs (Figure 1C). In the supernatants of unstimulated OASFs, PBEF protein was not detectable. However, in response to stimulation with poly(I-C), both RASFs and OASFs clearly showed up-regulated secretion of PBEF. Collectively, the results indicate that SFs secrete PBEF protein, and that the production of PBEF is up-regulated by TLR ligands, most notably by poly(I-C), as well as by the cytokines IL-1β and TNFα.

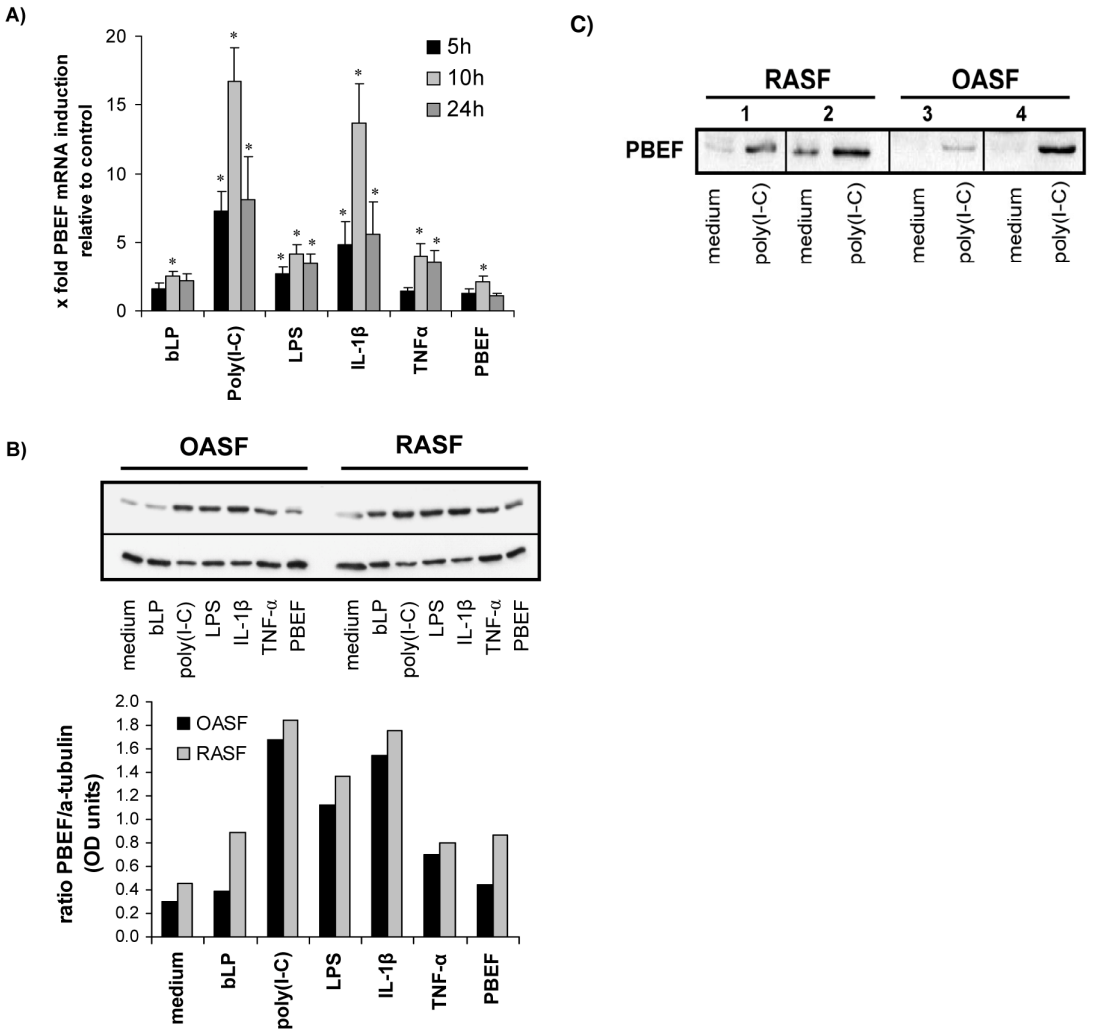

Figure 1. Pre-B cell colony-enhancing factor (PBEF) induction in rheumatoid arthritis synovial fibroblasts (RASFs) by Toll-like receptor (TLR) ligands and cytokines. A, RASFs (n = 5) were treated for 5, 10, and 24 hours with the indicated stimuli or were left untreated. Bars show the mean and SEM. *= *P* < 0.05, treated versus untreated cultures. B, Western blot analysis of PBEF expression in RASF and osteoarthritis SF (OASF) cell lysates, 24 hours following stimulation with the indicated TLR ligands and cytokines (α-tubulin served as a loading control). Protein levels were evaluated using densitometry. Results are representative of 3 individual experiments. C, Western blot analysis of secreted PBEF protein in supernatants of RASFs (n = 2) and OASFs (n = 2) stimulated for 24 hours with poly(I-C). bLP = bacterial lipoprotein; LPS = lipopolysaccharide; IL-1β= interleukin-1β; TNFα = tumor necrosis factor α; OD = optical density

Predominant expression of PBEF in RASFs in the synovial lining and at sites of invasion. Next, we analyzed the expression of PBEF in synovial tissue from patients with RA. In situ hybridization revealed pronounced expression of PBEF mRNA predominantly in the synovial lining and at sites of attachment and invasion of RASFs into cartilage or bone (Figure 2A). The expression of PBEF protein in RA synovia was also confirmed by immunohistochemistry, documenting abundant expression of PBEF in RA synovium (Figure 2B). PBEF expression was found to be highest at sites of invasion and in the synovial lining

layer, and it was detected to a lesser extent in the sublining and perivascular regions. Double-labeling revealed that PBEF was more frequently expressed in vimentin-positive SFs than in CD68-positive monocyte/macrophages (Figure 2C).

To study the association of PBEF levels with chronic joint inflammation, we compared the expression of PBEF protein in RA and noninflammatory OA synovial tissue. Analysis of OA synovial tissue sections revealed reduced expression of PBEF protein compared with that in RA synovial tissue, with PBEF being mainly expressed around small vessels (Figure 2D). Western blot analysis of total protein extracted from synovial tissue from 3 individual patients with RA and 3 patients with OA confirmed increased expression of PBEF protein in synovial tissue from the joints of patients with RA compared with patients with OA (Figure 2E).

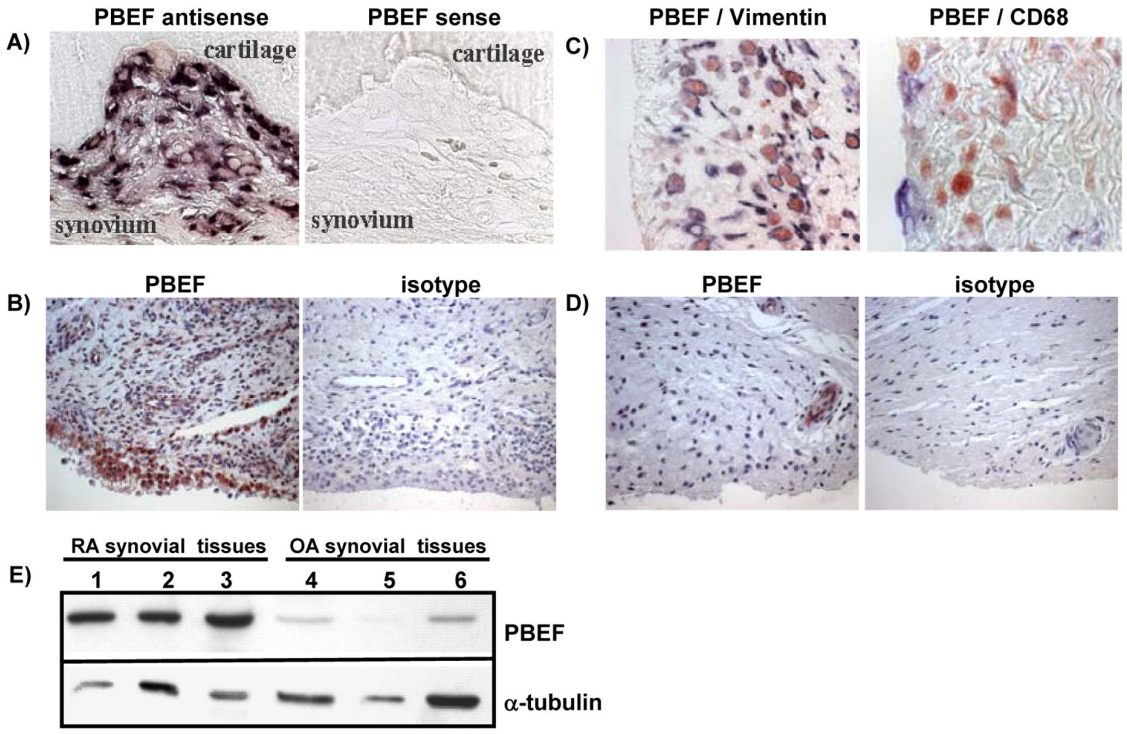

Figure 2. PBEF overexpression in synovial tissue samples from patients with RA. A, Representative section of RA synovial tissue specimens (n = 5) hybridized in situ with specific antisense RNA probes for PBEF mRNA. As negative control, tissue sections were hybridized with the sense probes. Cells expressing PBEF mRNA appear as dark blue. B, Representative section of RA synovial tissue (n = 10) stained for PBEF protein, with the corresponding tissue sections stained with isotype antibodies. PBEF protein appears as red. Nuclei were stained with hematoxylin. C, Double-labeling in RA synovial tissue. PBEF appears as red, and vimentin and CD68 appear as blue. D, Representative section of OA synovial tissue stained for PBEF (n = 5). Corresponding tissue sections were stained with isotype control antibodies. PBEF protein appears as red. Nuclei were stained with hematoxylin. E, Western blot showing the expression of PBEF protein in synovial tissue samples obtained from 3 patients with RA and 3 patients with OA; α-tubulin served as a loading control. (Original magnification × 400 in A and C; × 100 in B and D.) See Figure 1 for definitions.

Positive correlation of PBEF with the C-reactive protein (CRP) level and clinical disease activity in patients with RA. Because of the abundant expression of PBEF in the joints of patients with RA compared with that in the joints of patients with OA, we investigated whether serum and synovial fluid levels of PBEF might also reflect the severity of inflammation. Levels of PBEF were significantly higher in serum and synovial fluid samples from patients with RA compared with those in samples from patients with OA (Figure 3A). However, high variability in PBEF levels was observed among individual patients with RA. Using correlation analysis with the CRP level and PBEF, we analyzed whether these varying levels of PBEF might be associated with the degree of inflammation (Figure 3B). A significant positive correlation between the CRP level and PBEF in serum and in synovial fluid samples was observed. Additionally, the levels of PBEF in serum and synovial fluid and the Disease Activity Score in 28 joints (19) in individual patients with RA also showed a significant positive correlation (Figure 3C). Therefore, our results demonstrated that PBEF is associated with serum markers of inflammation as well as clinical disease activity in RA.

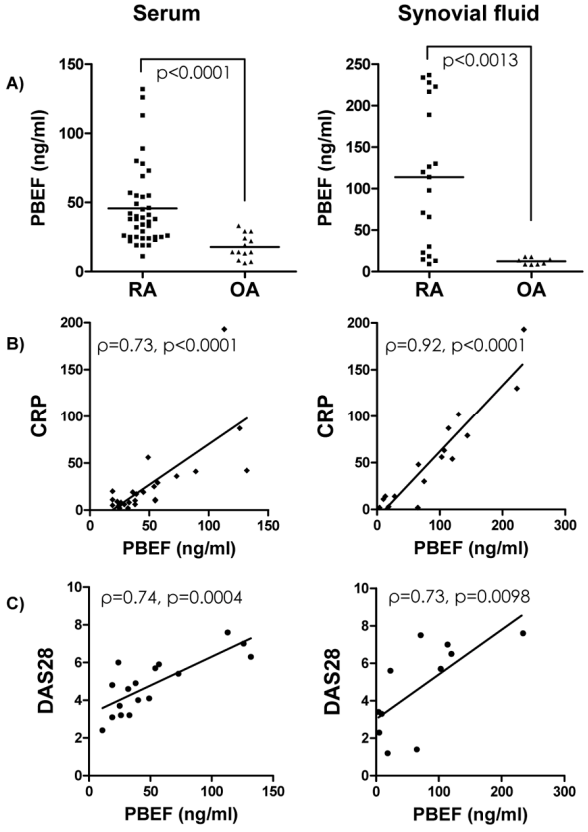

Figure 3. Correlation of elevated PBEF levels and disease severity in patients with RA. A, Serum and synovial fluid levels of PBEF in patients with RA and patients with OA, as measured by enzyme immunoassay. Horizontal bars show the means. B, Significant correlation of C-reactive protein (CRP) levels and PBEF concentrations in serum and synovial fluid from patients with RA. C, Significant correlation of the Disease Activity Score in 28 joints (DAS28) and PBEF concentrations in serum and synovial fluid from patients with RA. See Figure 1 for other definitions.

PBEF-induced production of IL-6, MMP-1, and MMP-3 in RASFs. To investigate the functional role of PBEF secreted in joints of patients with RA, RASFs were stimulated with increasing amounts of rHuPBEF for 24 hours. Recombinant human PBEF induced a dose-dependent increase in the levels of IL-6, MMP-1, and MMP-3. Already after stimulation with physiologic concentrations of rHuPBEF (50-200 ng/ml), IL-6 production was significantly up-regulated (Figure 4A). Similarly, RASFs treated with rHuPBEF showed significantly up-regulated expression of MMP-1 and MMP-3 mRNA upon incubation with 100 and 200 ng/ml of rHuPBEF (Figure 4B). To ascertain that the stimulatory effect was not attributable to contamination with endotoxin, RASFs were cultured in the presence of polymyxin. Polymyxin neutralized the stimulatory effect of 10 ng/ml of LPS, whereas the effect of PBEF on IL-6, MMP-1, and MMP-3 production remained unchanged (data not shown).

We additionally analyzed whether comparable results could be obtained by overexpressing PBEF in RASFs, using a eukaryotic expression vector. When a pcDNA3.1/PBEF/His expression construct was transfected into RASFs, the resulting protein could be demonstrated in the cell lysate by Western blotting (Figure 4C). Supernatants from RASFs obtained 48 hours after transfection with the PBEF/His construct showed significantly higher levels of IL-6 compared with that in RASFs transfected with the empty pcDNA3.1/His vector (Figure 4D). Additionally, levels of MMP-1 and MMP-3 were significantly higher in PBEF/His vector-transfected RASFs compared with those in controls (Figure 4E). These results demonstrated that PBEF induces proinflammatory cytokines and destructive enzymes in RASFs.

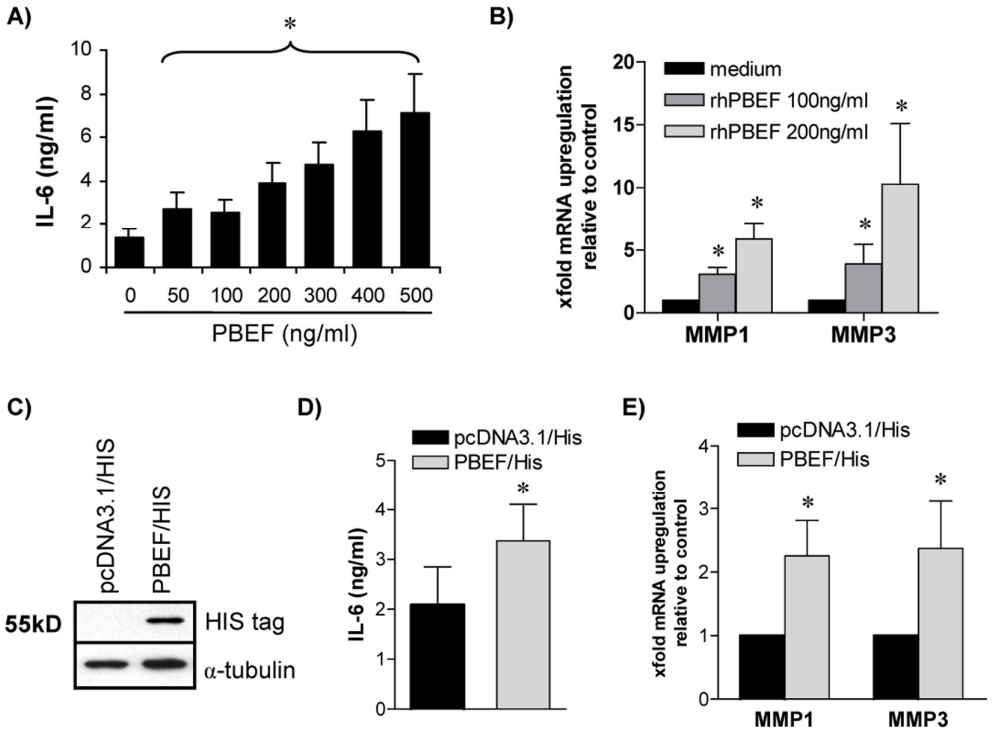

Figure 4. Up-regulation of proinflammatory cytokines and matrix-degrading enzymes in RASFs by recombinant human PBEF (rhPBEF) and PBEF overexpression. A, RASF cultures (n = 5) were incubated with the indicated concentrations of rhPBEF. Twenty-four hours following stimulation, levels of IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). B, RASF cultures (n = 5) were incubated with 100 ng/ml and 200 ng/ml of rhPBEF. Up-regulation of matrix metalloproteinase 1 (MMP-1) and MMP-3 mRNA was determined by real-time polymerase chain reaction. C, Western blot showing detection of PBEF with anti-His antibody at 55 kd in cell lysates from PBEF/His-transfected RASFs, but not from RASFs transfected with plasmid containing His alone (pcDNA3.1/His). D, Levels of IL-6 in supernatants (as measured by ELISA), following 24-hour incubation of RASF cultures overexpressing PBEF (n = 5). E, Up-regulation of MMP in RASF cultures overexpressing PBEF (n = 5) compared with that in mock-transfected RASFs (as measured by real-time polymerase chain reaction), following incubation for 24 hours. Bars show the mean and SEM. *= *P* < 0.05 versus control cultures. See Figure 1 for other definitions.

Reduction of basal and TLR ligand-induced IL-6, MMP-1, and MMP-3 levels by PBEF knockdown. PBEF is expressed in unstimulated RASFs, and we demonstrated that PBEF regulates IL-6, MMP-1, and MMP-3 production. Therefore, we investigated whether PBEF knockdown inhibits the characteristically high basal production of IL-6, MMP-1, or MMP-3 in RASFs. For this purpose, RASFs were transfected with PBEF-specific siRNA (siPBEF) or with nonspecific scrambled siRNA (scrambled) as a control. To ensure that responses to siPBEF transfection reflected PBEF knockdown, PBEF protein in cell lysates was quantified 72 hours after transfection. In 3 different RASF cultures analyzed, the inhibition of PBEF by specific siRNA was 69%, 59%, and 74%, respectively, as compared with that in controls (Figure 5A). Basal secretion of IL-6 was measured in the culture supernatants of siRNA-transfected RASFs (n = 6), 4, 6, and 24 hours after medium replacement. The basal

production of IL-6 was significantly lower in RASFs with down-regulated PBEF compared with controls at all time points analyzed. The most pronounced effect was seen 6 hours following medium replacement, with a mean ± SEM inhibition of 48.8 ± 7.2% (Figure 5B). Additionally, basal levels of MMP-1 and MMP-3 mRNA were down-regulated 4.7 ± 1.6-fold and 2.8 ± 0.6-fold, respectively, 48 hours following siPBEF transfection (Figure 5C).

In previous studies, we demonstrated that TLR-2, TLR-3, and TLR-4 ligands induce high amounts of cytokines and matrix-degrading enzymes in RASFs (3, 5, 20). In order to investigate whether PBEF is involved in the up-regulation of these effector molecules, we treated siPBEF-transfected RASFs with bLP, poly(I-C), and LPS, and analyzed the induction of IL-6, IL-8, MMP-1, and MMP-3, 24 hours after TLR ligand stimulation. PBEF knockdown significantly inhibited the up-regulation of all measured effector molecules (Figures 5D and E). These data indicated that PBEF is implicated in basal as well as TLR ligand-induced production of proinflammatory cytokines and matrix-degrading enzymes.

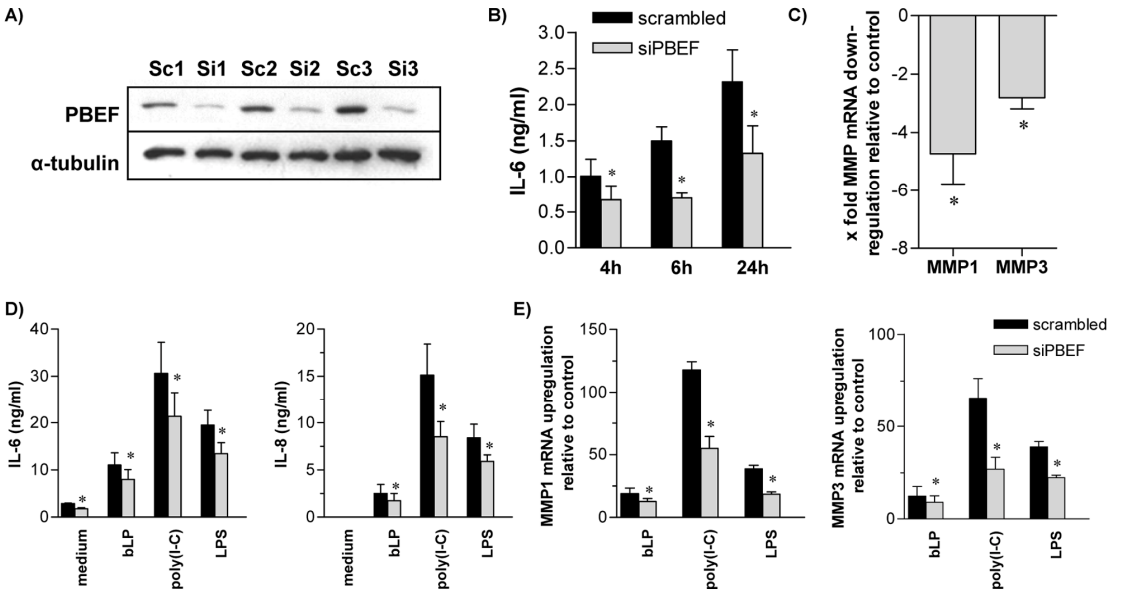

Figure 5. Inhibition of basal and TLR ligand-induced cytokine and matrix metalloproteinase (MMP) production by PBEF knockdown. A, RASFs transfected with PBEF-specific small interfering RNA (siRNA; Si1-3) and nonspecific scrambled siRNA (Sc1-3) were subjected to Western blot analysis for PBEF; α-tubulin served as a loading control. B, RASF cultures (n = 5) with down-regulated PBEF and corresponding control cultures were incubated for 4, 6, and 24 hours, followed by measurement of IL-6 in the culture supernatant. C, MMP-1 and MMP-3 mRNA up-regulation in siPBEF-transfected RASFs compared with scrambled siRNA-transfected RASFs was measured by real-time polymerase chain reaction (n = 5 cultures for 5 different patients). D, RASFs with down-regulated PBEF and corresponding controls were stimulated with the indicated TLR ligands for 24 hours, and levels of IL-6 and IL-8 were determined in the culture supernatants. E, RASFs with down-regulated PBEF and corresponding controls were stimulated with the indicated TLR ligands for 24 hours. MMP-1 and MMP-3 mRNA up-regulation in siPBEF- and scrambled siRNA-transfected RASFs compared with untreated RASFs is shown. Bars show the mean and SEM. *= $p < 0.05$ versus control-transfected RASFs.

PBEF induction of TNFα and IL-6 in primary human monocytes. It has been demonstrated that primary blood monocytes express PBEF (10). However, it has not been determined whether monocytes are responsive to PBEF. Because we observed high levels of PBEF in serum obtained from patients with RA, we analyzed whether human primary blood monocytes are activated either by rHuPBEF or by overexpression of PBEF. Monocyte cultures treated with 50 ng/ml of rHuPBEF, a concentration that corresponds to the level of PBEF in serum from patients with RA, showed significantly higher production of IL-6 and TNFα as compared with that in untreated cultures. The stimulatory effect of rHuPBEF on monocytes was dose dependent (Figures 6A and B). Similarly, overexpression of PBEF in monocytes resulted in a 5.0 ± 1.5-fold increase in TNFα secretion (Figure 6C). Thus, PBEF is a potent activator of human monocytes, inducing the production of key proinflammatory cytokines such as IL-6 and TNFα.

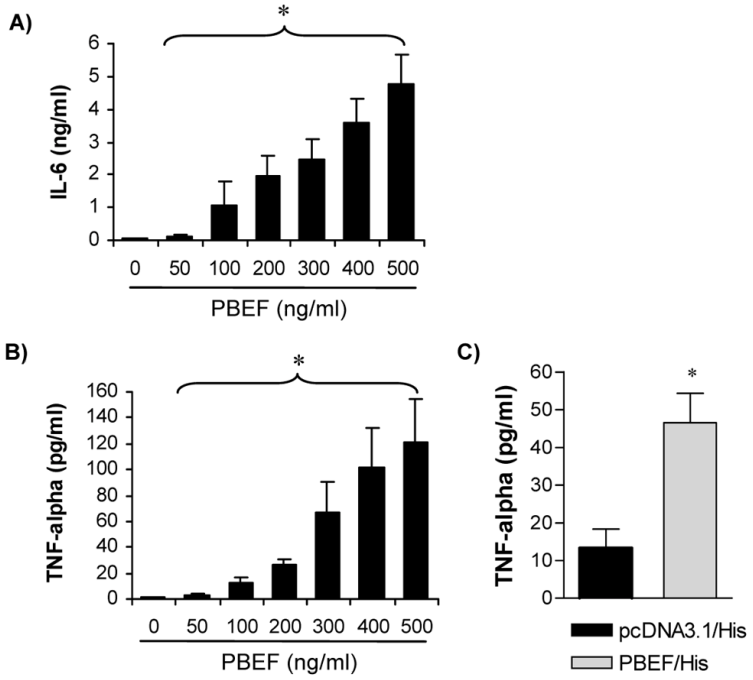

Figure 6. Up-regulation of key proinflammatory cytokines in human primary monocytes by recombinant human pre-B cell colony-enhancing factor (rHuPBEF) and PBEF overexpression. Monocytes were incubated with the indicated concentrations of exogenously added rHuPBEF (n = 5 cultures for 5 different patients). A and B, Twenty-four hours following stimulation, levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were measured in the culture supernatants. C, Monocytes transfected with PBEF/His expression constructs and monocytes transfected with plasmids containing His alone (pcDNA3.1/His) were incubated for 24 hours. Levels of TNFα were measured in the culture supernatants (n = 5). Bars show the mean and SEM. *= p < 0.05 versus control cultures.

PBEF stimulates RASF via NF-κB and AP-1. The activation of the transcription factors NF-κB and AP-1 is a principal step for the initiation and maintenance of inflammatory responses. Therefore we studied whether rhPBEF activates NF-κB and AP-1 signalling pathways in RASF. Nuclear extracts of RASF stimulated with rhPBEF (100 ng/ml) were subjected to EMSA. 90 minutes following stimulation the activation of RASF resulted in translocation of NF-κB and AP-1 from the cytoplasm to the nucleus (Supplementary figure 1A,B). Therefore PBEF has the capacity to activate two major transcription factors such as NF-κB and AP-1.

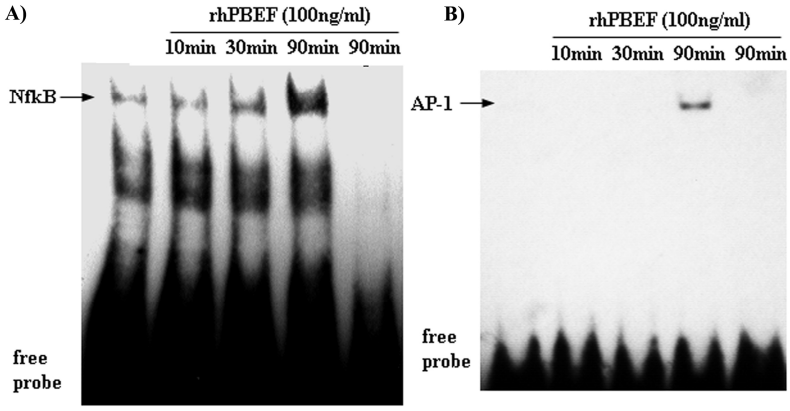

Figure 7: PBEF activates the transcription factors NF-kB and AP-1. Binding of nuclear extracts from RASF stimulated with rhPBEF to NF-kB (A) and AP-1 (B) specific binding sites, assessed by EMSA. *Lane1:* Binding of nuclear extracts from untreated RASF. *Lanes 2,3,4:* Binding of nuclear extracts from RASF stimulated for 10, 30 and 90 minutes with rhPBEF, respectively. *Lane 5:* Nuclear extract as in *lane 4* after preincubation with an excess of competitive binding unlabeled oligonucleotides as a negative control.

Discussion

In the present study, we demonstrated induction of the adipocytokine PBEF in RASFs via TLR stimulation as well as by stimulation with IL-1β and TNFα. Furthermore, we observed potent proinflammatory and matrix-degrading activities of PBEF and showed that in patients with RA, levels of PBEF correlate with the severity of inflammation.

Using a subtractive hybridization assay of RASFs stimulated with poly(I-C), we found PBEF to be up-regulated. However, PBEF induction in RASFs is not restricted to TLR-3 activation, as shown by stimulation experiments with other TLR ligands and proinflammatory cytokines that can be found in the joints of patients with RA (1). Interestingly, PBEF itself induces its own production, indicating the existence of a positive feedback-regulating mechanism. Expression and up-regulation of human PBEF has previously been documented in neutrophils by IL-1β and LPS, in amniotic cells by TNFα, in monocytic cells by nitric oxide, in

adipocytes by hypoxia, and in RASFs by IL-6 trans-signaling (10, 11, 13, 21-23). Taken together, these data show that signaling pathways of the innate immune system have a strong regulatory effect on the expression of PBEF in a variety of cell types.

Our study demonstrates accumulation of PBEF in the joints of patients with RA and identifies SFs as the major PBEF-producing cells in the rheumatoid synovium. PBEF expression is predominantly localized at the site of invasion into cartilage and in the synovial lining. This staining pattern may reflect the local availability of PBEF-stimulating agents such as cytokines and TLR ligands in the lining layer. Additionally, hypoxic conditions that are most marked at the site of invasion into cartilage may drive local PBEF expression, as has been shown previously in adipocytes (22).

It is known that IL-6 exerts stimulatory effects on T cells and B cells, thus favoring chronic inflammatory responses, whereas MMPs have been closely linked to the progressive destruction of articular cartilage in rheumatoid joints. A hallmark of RASFs is the high basal production of IL-6 and MMPs (24). We showed that PBEF has an important role in the regulation of these key proinflammatory and matrix-degrading molecules. Recombinant human PBEF induces the expression of IL-6 and MMPs in RASFs, suggesting that secreted PBEF contributes to the local inflammatory and destructive processes in arthritic joints. Moreover, in our experiments, down-regulation of PBEF in RASFs by siRNA not only decreased basal IL-6, MMP-1, and MMP-3 levels but also significantly inhibited TLR ligand-induced production of cytokines and destructive enzymes. TLRs were shown to be key players in inflammatory and destructive processes in RA (3, 4, 20, 25). TLR ligands of microbial origin as well as endogenous TLR ligands were demonstrated to be present in RA synovial fluid as possible drivers of inflammatory processes (1, 26). Our findings suggest that the stimulation of RASFs by TLR ligands is at least partially dependent on PBEF expression. Therefore, targeting PBEF not only might counteract its direct stimulatory effect on RASFs but also reduces TLR-driven proinflammatory and destructive responses.

Peripheral blood monocytes have been shown to be a source of PBEF in the blood circulation. Our study shows that primary human monocytes are responsive to PBEF, suggesting that PBEF acts in an autocrine manner to increase serum levels. Notably, activation of monocytes by PBEF results in the production TNFα, a key cytokine in the pathogenesis of RA (27). Moreover, PBEF present in synovial fluid might trigger chronic inflammation not only via

induction of proinflammatory cytokine production by RASFs but also by a direct antiapoptotic effect on neutrophils (10). Neutrophils are abundant in the synovial fluid of patients with RA and are less susceptible to TNFα-induced apoptosis than are blood neutrophils. In this regard, it has been shown that synovial fluid from patients with RA exhibits an antiapoptotic effect on neutrophils (28).

The possible mechanisms by which PBEF exerts its proinflammatory effects in the arthritic joint are incompletely understood. The identification of PBEF in the visceral fat added PBEF to a growing list of adipocytokines with potent effects on immunity and inflammation in addition to their metabolic activity (9). Leptin, adiponectin, resistin, and most recently PBEF have been shown to be up-regulated in patients with RA compared with healthy control subjects (14, 29, 30). PBEF mimics insulin signaling by binding to the insulin receptor with an affinity similar to that of insulin but does not share the binding site with insulin on the insulin receptor. In contrast, previous studies have shown intracellular expression of PBEF and have demonstrated that PBEF is a nicotinamide phosphoribosyltransferase (7, 8). Overexpression of PBEF in human vascular SMCs induced enhanced survival by its regulatory effect on NAD-dependent deacetylase activity. Moreover, it has been shown that subcellular localization of PBEF is dependent on the cell cycle, suggesting a role for PBEF in cell cycle regulation (31). Using immunohistochemical analysis, we observed nuclear and cytoplasmic expression of PBEF. Whether the effects provoked by PBEF are dependent on binding to the insulin receptor or an as yet unknown receptor, or alternatively by its enzymatic activity, needs to be determined in further studies.

Elevated levels of PBEF in serum and synovial fluid from patients with RA have been observed in this and previous studies (13,14). Our new finding of a strong correlation of PBEF with markers of inflammation such as the CRP level provides support for an important role of this cytokine in inflammatory reactions. This is further underscored by the correlation of PBEF concentrations with scores for clinical disease activity (comprising the tender and swollen joint counts and the erythrocyte sedimentation rate) (32). These findings suggest that PBEF is a marker of the severity of inflammation in patients with RA.

Taken together, our results suggest that PBEF plays a key role as a mediator of innate immune pathways in chronic synovial inflammation and joint destruction, and identify this adipocytokine as a possible therapeutic target for the treatment of RA.

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CHAPTER 5

Abundant expression of the IL-23 subunit p19 but low levels of bioactive IL-23 in the rheumatoid joint

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Abstract

Objective. IL-23, composed of a p19 and a p40 subunit, is suggested to play key roles in rheumatoid arthritis (RA), dependent on the promotion and proliferation of IL-17-producing Th17 T-cells. However, previous studies on IL-23 expression in human tissues were based on the subunit p19 only. We aimed to study the expression and regulation of both IL-23 subunits, p19 and p40, in RA compared to osteoarthritis (OA) patients.

Methods. The expression of p19 and p40 in synovial tissues was analyzed by in-situ hybridization and/or immunohistochemistry. IL-23 in RA and OA synovial fluids and sera was determined by ELISA. TLR-dependent induction of p19, p40 and bioactive IL-23 was determined in RASF, monocytes and MDDC by RT-PCR, Real-time PCR, Western-blot and functional-assays.

Results. The p19 subunit was abundantly expressed in RA but not in OA synovial tissues. RASF in the synovial lining layer and at the site of invasion showed the most prominent expression of p19 but not heterodimeric IL-23. Correspondingly, soluble IL-23 was found at very low levels in synovial fluids and sera of patients with RA. By in vitro experiments, we confirmed that TLR activated RASF expressed p19 but not p40, in contrast to monocytes and MDDCs, which produced IL-23 following TLR stimulation.

Conclusion. The TLR-dependent induction of p19 but not p40 in RASF and the abundant expression of p19 along with the weak expression of IL-23 in RA patients, gives strong evidence that p19 does not necessarily indicate the presence of active IL-23 as it has been proposed so far.

Introduction

Interleukin-23 (IL-23) is a heterodimeric cytokine composed of a p19 and a p40 subunit which is shared with IL-12. The p19 subunit has been identified by computational screening of the IL-6 helical cytokine family. Purified p19 has no biological activity in vitro, whereas p19 combined with the p40 subunit forms the heterodimeric and biologically active cytokine IL-23 [1]. The major source of IL-23 are macrophages and dendritic cells. Toll-like receptor agonists and interactions with T cells (CD40/CD40L interaction) were shown to trigger the induction of bioactive IL-23 [2-4]. IL-23 is related to IL-12 but has functionally distinct properties. IL-12 is composed of the p40 and the p35 subunit, which has an overall sequence identity of approximately 40% to the p19 subunit. In contrast to IL-12, IL-23 does not promote the development of IFN-gamma producing Th1 CD4+ cells, but is one of the essential factors required for the proliferation and maintenance of a recently described IL-17 producing CD4+ T-cell subset, named Th17 [5]. Expression of IL-17 has been detected in sera and tissues of patients with various autoimmune diseases, including rheumatoid arthritis (RA), multiple sclerosis and systemic lupus erythematosus [6-8].

RA is a chronic inflammatory disease that is characterized by the destruction of articular cartilage and bone. RA synovial fibroblasts (RASF) play a major role in the destruction of the joint by secreting matrix degrading enzymes. Moreover, a hallmark of RA is synovial hyperplasia that is caused by the proliferation of resident RASF and by the accumulation of inflammatory immune cells including B cells, T cells and macrophages. IL-23 is suggested to be essential in autoimmune inflammation in joints as p19 and p40 deficient mice exhibit reduced severity of collagen induced arthritis [9]. In humans, levels of p19 as well as p40 were shown to be elevated in synovial fluids and sera of patients with RA compared to osteoarthritis (OA) [10, 11]. These data suggested high expression of IL-23 in joints of RA patients even though the presence of heterodimeric IL-23 has not been ascertained so far.

There is mounting evidence for an activation of toll-like receptor (TLR) signalling pathways in RA. We and others have shown in previous reports elevated TLR2,3 and 4 levels in synovial tissues of patients with RA compared to OA. Furthermore, RASF stimulated with TLR2, 3 and 4 ligands produce high amounts of cytokines, chemokines and matrix metalloproteinases that are characteristically found in joints of RA patients. Regarding the induction of IL-23 in RASF, it has been demonstrated that the p19 subunit is upregulated by

IL-1β or IL-17 [10, 12]. However, whether RASF have the potential to produce heterodimeric IL-23 after TLR activation remains to be determined.

The purpose of our study was to analyze the presence of heterodimeric IL-23 in RA and OA synovial tissues by immunohistochemistry and to determine levels of soluble IL-23 in RA and OA synovial fluids and sera by enzyme-linked immunoabsorbent assay. Furthermore, we analyzed the induction of both IL-23 subunits, p19 and p40, in RASF, human primary blood monocytes and monocytes derived dendritic cells (MDDCs) after stimulation with TLR2, 3 and 4 ligands.

Materials and Methods

Patients and tissue preparation. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and OA, after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASF and OASF were isolated from synovial tissues, digested by collagenase, and used after passages 4 to 8 as described [13]. To obtain tissue sections, synovial specimens were fixed in paraformaldehyd and embedded in paraffin. Sera and synovial fluids from patients with RA and OA were collected, centrifuged and stored at -80° until analysis. Before analysis, synovial fluid samples were pre-treated for 1 hour at 37°C with 1 mg/ml of hyaluronidase (Fluka, Buchs, Switzerland). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [14].

In situ hybridization. IL-23 sense and IL-23 antisense probes for in situ hybridization (ISH) were prepared according to methods previously described. The primer sequences were as follows: upper primer 5'-CTA TCA GGG AGC AGA GAA G-3'; and lower primer, 5'- ACT AGT GGG ACA CAT GGA T-'3. [15]. ISH was performed as described by Kriegsmann et al.[16].

Immunohistochemistry. After the detection of IL-23 mRNA in RA synovial tissues by ISH, subsets of synovial cells expressing p19 were analyzed by double labeling the p19 mRNA stained sections with monoclonal mouse anti-human CD68 or anti-human vimentin antibodies (2µg/ml, Dako, Glostrup, DK), respectively. Bound mouse primary antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Europe, Soham, UK). HRP labelled cells were visualized using AEC (3-

Amino-9-Ethylcarbazol) substrate-chromogen (Dako).In order to analyze IL-23 protein expression, consecutive synovial tissue sections derived from patients with RA or OA were stained for p19 or/and p40. The catalyzed signal amplification (CSA) system for mouse primary antibodies was used according to the manufacturers instruction (Dako). Briefly, tissue sections were deparaffinized and pretreated with target retrieval solution. Endogenous avidin-binding activity was blocked with the avidin/biotin blocking system and endogenous peroxidase was blocked with 1.5% H_2O_2. After blocking unspecific IgG binding with protein block solution, sections were incubated for 15 min with mouse anti-human p19 antibodies (10ug/ml, BioLegend, San Diego, CA) or mouse anti-human p40 antibodies (10µg/ml, AbD Serotec, Germany). Sections were then incubated with biotinylated link antibodies, Streptavidin-biotin complex solution, amplification reagent and streptavidin-peroxidase. HRP-labelled cells were visualized using DAB (3,3'-diaminobenzidine tetrahydrochloride) and Histo-green substrate-chromogen. In control experiments, isotype matched mouse IgG were used instead of the primary antibodies. Tissues were counterstained with hematoxylin.

Stimulation assays with rheumatoid arthritis synovial fibroblasts. RASF were cultured in DMEM (Gibco, Basel, Switzerland) supplemented with 10% FCS and stimulated with the following agents: polyinosinic-polycytidylic acid (poly(I-C), 20 µg/ml; Invivogen, San Diego, CA), lipopolysaccharide from *E. coli* (LPS, 100 ng/ml; List Biologicals, Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (bLP, 300 ng/ml, Invivogen).

Isolation and stimulation of monocytes and generation of monocyte derived dendritic cells. Peripheral blood mononuclear cells (PBMCs) were isolated from Buffy coats of healthy volunteers with Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. Peripheral blood monocytes were positively separated from PBMCs with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers protocol. CD14+ cells were transferred in 12-well plates, cultured in RPMI-1640 supplemented with 5% FCS and stimulated with bLP (300 ng/ml), poly(I-C) (10ug/ml) or LPS (100ng/ml) for 24h. For the generation of monocyte derived dendritic cells (MDDCs), CD14+ cells were cultured for 6 days in RPMI-1640, supplemented with 10% FCS, interleukin 4 (IL-4, 500 U/ml; R&D Systems, Abington, UK) and granulocyte monocyte-colony stimulating factor (GM-CSF, 800 U/ml; R&D Systems). Fresh complete culture medium was added after 3 days. At day 6 the immature MDDCs (iMDDCs) were harvested.

To generate mature MDDCs, iMDDCs were transferred to new 12 well culture plates in fresh complete culture medium and stimulated for 24h with bLP (300 ng/ml), poly(I-C) (10ug/ml) or LPS (100ng/ml). Expression of cell surface markers (CD14, CD86 and CD83) on iMDDCs and mMDDCs was measured by fluorescence activated sell sorter (FACS) analysis in order to confirm corresponding phenotypes.

Real-time PCR. Total RNA from cultured RASF, monocytes and MDDC was isolated with the RNeasy MiniPrep Kit including treatment with RNase-free DNase (Qiagen, Basel, Switzerland) and reverse transcribed using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). Non reverse transcribed samples were used as negative controls. Quantification of p19 and p40 mRNA was performed by Taqman Real-time PCR using the ABI Prism 7700 Sequence Detection system (Applied Biosystems). The following validated TaqMan® gene expression assays (Applied Biosystems) were used: IL23A (p19) (Hs00372324_m1) and IL12B (p40) (Hs00233688_m1). The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle (Ct) method for relative quantification as described by the manufacturer.

Conventional RT-PCR. Total RNA was isolated as described above for Real-time PCR and reverse transcribed using oligo(dT) and moloney murine leukemia virus (MuLV) reverse transcriptase (both Invitrogen, Switzerland). Conventional PCR was performed on a GenAmp PCR System 9700 (Applied Biosystems, Rotkreuz, Switzerland) with the following primer pairs and protocols. p19: forward primer 5'- CTA TCA GGG AGC AGA GAA G -3', reverse primer 5'- ACT AGT GGG ACA CAT GGA T -3'; p40 forward primer 5'-ATG TCG TAG AAT TGG ATT GG -3', reverse primer 5'-AGG TGA AAC GTC CAG AAT AA -3'; βmicroglobulin forward primer 5'-AAG ATT CAG GTT TAC TCA CGT C-3', reverse primer 5'-TGA TGC TGC TTA CAT GTC TCG-3'. 5min 94°C; 30 cycles 30s at 94°C, 30s at Tm (Tm 54°C for p19, Tm 54°C for p40, Tm 56°C for β-microglobulin), 30s at 72°C; and a final elongation of 5min with 72°C. Reaction products were separated on a 1% agarose gel and signals were visualized using ethidium bromide. As a negative control, PCR was carried out in the absence of cDNA for each set of primers.

Enzyme linked immunoabsorbant assay (ELISA) and enzyme immunoassay (EIA). IL-23 heterodimer was detected using a human IL-23 ELISA Kit (eBioscience, San Diego, CA) according to the manufacturers instructions. Absorption was measured at 450 nm and data

were analyzed using Revelation v4.22 software (Dynex Technologies, Denkendorf, Germany).

Western blot. RASF were stimulated for 24h with TLR ligands before cells were lysed in Laemmli-Buffer. Proteins were separated on 10% SDS-polyacrylamide gel and blotted on Protran nitrocellulose transfer membrane (Schleicher & Schüll, Dassel, Germany). Membranes were probed with anti-p19 antibodies (0.5 µg/ml, BioLegend) and detected with HRP-conjugated second antibodies using the ECL Western blotting detection system (Amersham Pharmacia Biotech). Blots were stripped and reprobed with monoclonal mouse anti human α-Tubulin (Sigma) antibodies to confirm similar loading of the gels.

Results

Abundant IL-23 p19 mRNA expression in RA synovial tissues. To investigate whether IL-23 might play a role in the pathogenesis of RA we analyzed the presence and distribution of the IL-23 subunit p19 in the rheumatoid joint. Expression of p19 was assessed by in situ hybridization with an anti-p19 mRNA probe using paraffin embedded RA synovial tissue sections (n=5). We observed p19 mRNA positive cells thoroughout the synovium. The most marked expression of p19 mRNA was found in the synovial lining layer as well as at sites of invasion into cartilage. (Figure 1A, C). Control sections hybridized with sense- p19 mRNA probe showed no signal (Figure 1B, D). Furthermore, double staining with cell type specific markers revealed that p19 positive cells expressed the fibroblast marker vimentin or the macrophage marker CD68 (Figure 1E, F), documenting strong p19 mRNA expression by RA synoviocytes.

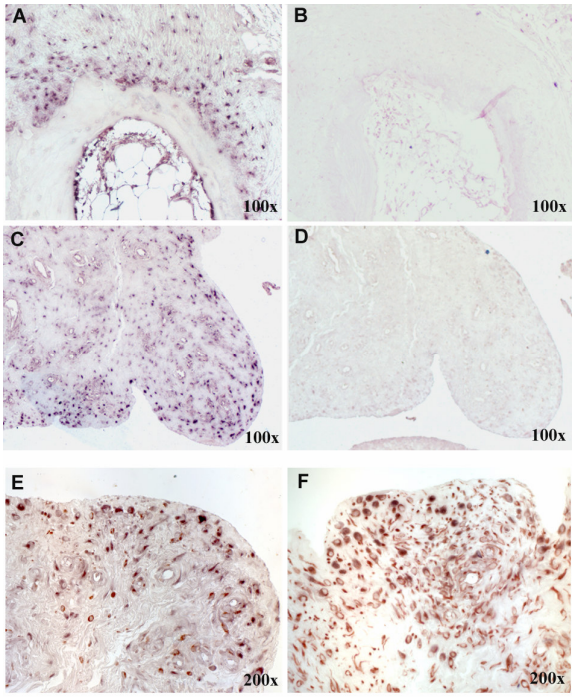

Figure 1: In RA synovial tissues p19 mRNA is expressed by synoviocytes. Representative sections of RA synovial tissues (n=5) hybridized in situ with specific antisense RNA probes for p19 mRNA. p19 mRNA was detected most marked at sites of invasion into cartilage (A) and in the synovial lining layer (C). Cells expressing p19 mRNA appear in dark blue. As negative control, tissue sections were hybridized with the corresponding p19 sense probes (B, D). Sections stained for p19 mRNA were further labeled by immunohistochemical analysis with anti-CD68 antibodies as macrophage markers (E) or anti-vimentin antibodies as fibroblast markers (F). CD68 or vimentin positive cells appear in red colour.

Differential p19 and p40 protein expression in RA and OA synovial tissues

IL-23 is a heterodimer consisting of the p19 and the p40 subunit, the latter being shared with IL-12. In order to examine whether p19 protein expression co-localizes with p40 protein expression we performed immunohistochemical analysis for p19 and p40 on consecutive RA synovial tissue sections (n=6). In RA synovial tissues p19 protein was abundantly expressed and the distribution corresponded to the presence of p19 mRNA as detected by in situ hybridisation (Figure 2B). In contrast to the broad expression of the p19 subunit, p40 protein expression was found to be restricted to few cell clusters scattered in the synovium (Figure 2C). These results revealed, that most p19 expressing synoviocytes do not co-express the p40 subunit. Control sections incubated with matched isotype antibodies showed no signal (Figure 2A,D). In addition we analyzed the expression of p19 and p40 in synovial tissues derived from patients with osteoarthritis (OA). Both subunits, p19 and p40, were not, or very weakly expressed in OA synovial tissues (n=5) (Figure 2E, F). Thus, the subunits p19 and p40 might be implicated in the pathogenesis of RA but not OA.

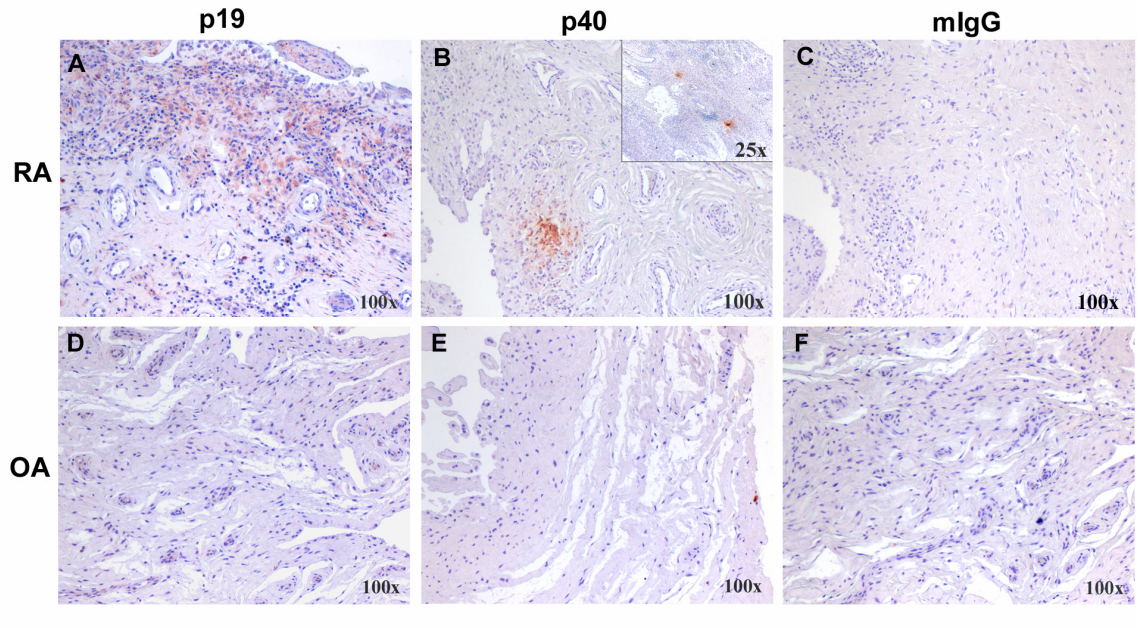

Figure 2: In RA synovial tissues p19 and p40 protein is abundantly and differentially expressed. Representative sections of RA synovial tissues (n=6) stained for p19 (A) or p40 protein (B) and OA synovial tissues (n=5) stained for p19 (D) or p40 protein (E) using immunohistochemistry. Staining with isotype matched mouse antibodies served as negative control (C, F). p19 and p40 protein appear in red colour. Nuclei were stained with hematoxylin.

Presence of heterodimeric IL-23 in RA synovium. We observed distinct staining patterns for p19 and p40 in synovial tissues derived from patients with RA. To address the question whether heterodimeric IL-23 is present in joints of RA patients we performed immunohistochemical double stainings for p19 and p40 on RA synovial tissue sections (n=6). The majority of synoviocytes in the synovial lining layer as well as in the sublining were single positive for p19 (Figure 3B,C). In addition we also detected p40 single positive cells (Figure 3B). However, within some p40 positive cell clusters we found p19/p40 double positive cells (Figure 3C). Control sections incubated with matched isotype antibodies showed no signal (Figure 3A). These results demonstrate that IL-23 is expressed in RA synovium but only by a small number of cells. Furthermore, we show that the majority of cells did not express p40 and therefore no heterodimeric IL-23.

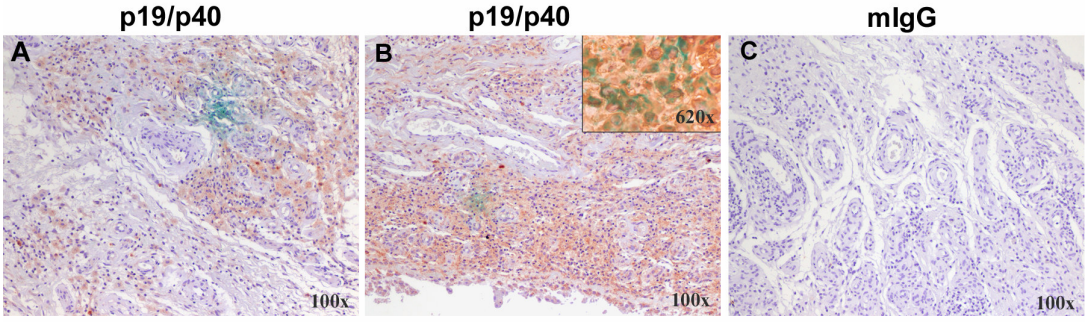

Figure 3: The heterodimeric cytokine IL-23 is present in RA synovial tissues. Representative sections of RA synovial tissues (n=6) double labeled for p19 and p40 protein using immunohistochemical analysis (A, B). Staining with isotype matched mouse antibodies served as negative control (C). p19 appears in brown, p40 in green colour. Nuclei were stained with hematoxylin.

Levels of soluble IL-23 in RA and OA synovial fluids and sera. The concentration of the shared IL-12/IL-23 subunit p40 was reported to be elevated in synovial fluids of RA patients compared to controls [11, 17] and high levels of soluble p19 protein in RA synovial fluid and serum samples were demonstrated recently by Kim et al. [10]. By immunohistochemical analysis we detected only a small number of p19/p40 double positive cells in RA synovial tissues. To assess the concentrations of soluble IL-23 in synovial fluids and sera from patients with RA and OA we performed ELISA specific for the IL-23 heterodimer (Figure 4). In only 5 out of 28 synovial fluids derived from patients with RA and in only 1 out of 10 synovial fluids of patients with OA we were able to detect concentrations of IL-23 above the detection limit (range: 16-29pg/ml IL-23). Similarly, we also detected only small amounts of IL-23 in 9 out of 24 RA sera and in 8 out of 22 OA sera (range: 16-114pg/ml IL-23). No statistically significant difference of IL-23 levels between RA and OA synovial fluids and sera were observed.

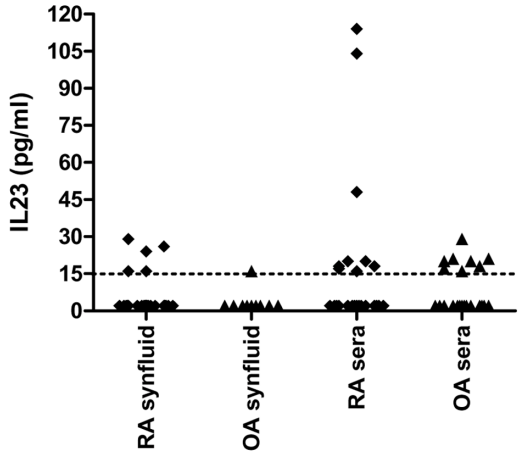

Figure 4: Low levels of soluble IL-23 in synovial fluids and sera derived from patients with RA. Concentrations of heterodimeric IL-23 in RA synovial fluids (n=28), OA synovial fluids (n=10), RA sera (n=22) and OA sera (n=18), determined by ELISA. Each dot represents the average amount of soluble IL-23 from duplicates of the same individual. The detection limit of the ELISA (16 pg/ml IL-23) is indicated.

Regulation of p19 and p40 expression by TLR ligands. As RA synoviocytes express elevated levels of TLR2, 3 and 4, we assessed TLR dependent regulation of the IL-23 subunits p19 and p40 in different cell types. RASF, monocytes or monocyte derived dendritic cells (MDDCs) were stimulated with the TLR2 ligand bLP, the TLR3 ligand poly(I-C) and the TLR4 ligand LPS. 24h following stimulation we determined the presence and the induction of the two IL-23 subunits by conventional RT-PCR as well as by quantitative real-time PCR. RASF did not express p19 mRNA constitutively (Figure 5A). However after stimulation with TLR2, 3, and 4 ligands p19 mRNA was found to be induced in RASF (Figure 5A, B). The most prominent induction was seen after TLR3 activation with poly(I-C) (24.5 ±2.1 fold upregulation relative to unstimulated cultures, n=5). Conversely, p40 mRNA was neither expressed in unstimulated nor in TLR ligand stimulated RASF. In two RASF cultures derived from individual patients the induction of p19 protein by TLR ligands was confirmed on protein levels by Western blot analysis (Figure 5C).

In primary monocytes both IL-23 subunits were inducible to similar levels by bLP as well as by LPS (Figure5 A,B). However no constitutive mRNA expression was detected for p19 nor p40 (Figure 5A). Because monocytes do not express TLR3, neither p19 nor p40 mRNA was detected after stimulation with poly(I-C). MDDCs constitutively expressed the p40 subunit (Figure 5A). In addition p40 mRNA was strongly upregulated following stimulation with bLP and LPS as well as with poly(I-C) (Figure 5A,B). The p19 subunit was not expressed in unstimulated MDDCs but was induced after stimulation with the TLR2, 3 and 4 ligands. Thus, TLR activated monocytes and MDDCs have the potential to induce both subunits necessary for the production of bioactive IL-23, whereas TLR activated RASF express the p19 subunit only.

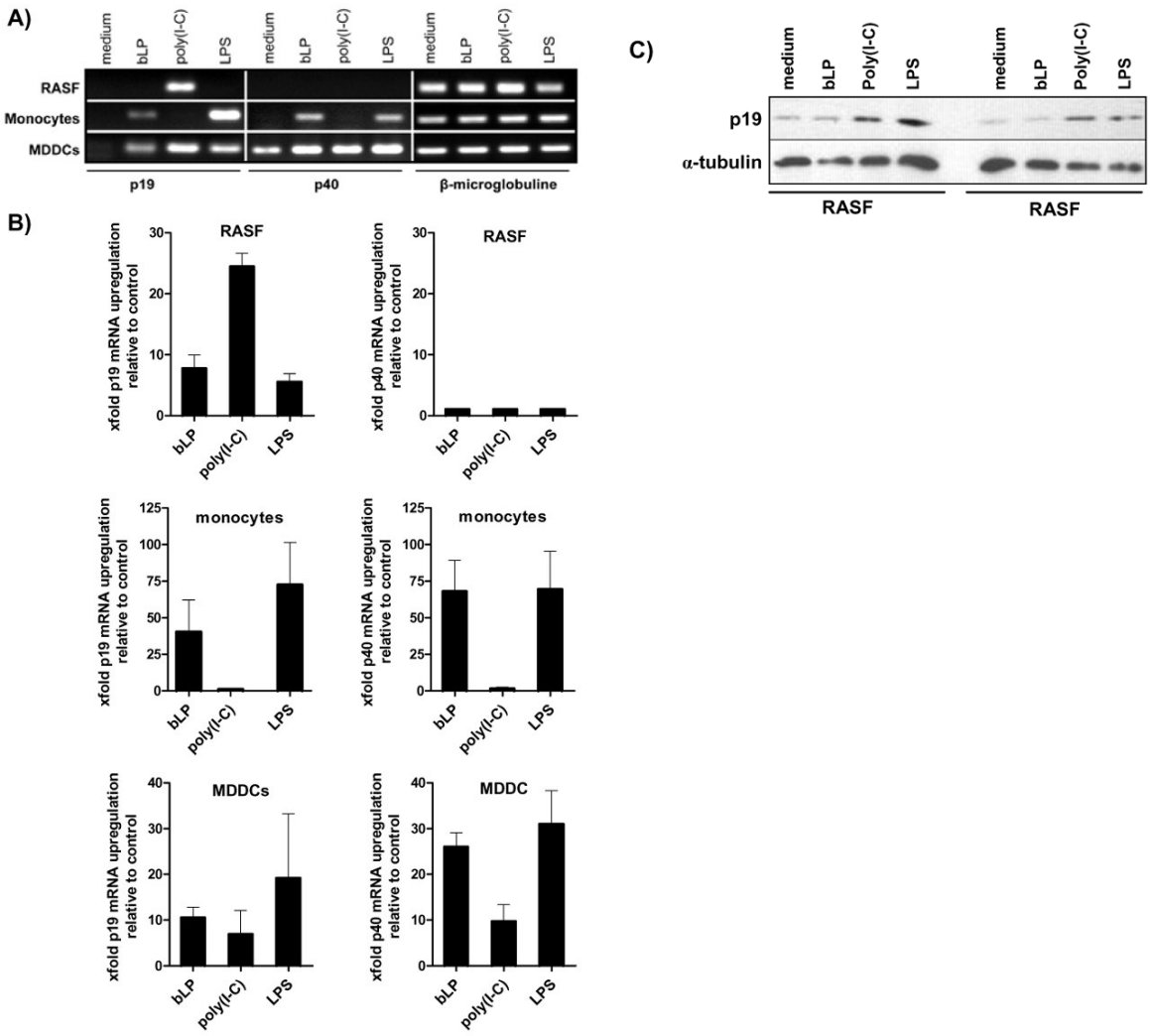

Figure 5: Differential expression and induction of the IL-23 subunits, p19 and p40, by TLR ligands. RASF cultures (n=5), monocyte cultures (n=3) and MDDC cultures (n=3) were stimulated for 24h with the indicated TLR ligands. Conventional PCR (A) or Real-time PCR (B) was performed in order to analyze p19 and p40 mRNA expression. (A) One representative experiment out of five is demonstrated. (B) The mean ± SEM p19 or p40 mRNA upregulation relative to untreated cultures is shown. C) Western blot analysis of p19 expression in two individual RASF cultures, 24h following stimulation with the indicated TLR ligands. a-tubulin served as a loading control.

Activated Monocytes but not RASF secrete bioactive IL-23

It has been demonstrated that stimulation of CD8+ T-cells with rhIL-23 results in the production of IL-17 [18]. To assess whether monocytes or RASF secrete biologically active IL-23, we stimulated polyclonally activated CD8+ T-cells with supernatants of TLR ligand stimulated monocytes and RASF and measured IL-17 production. Stimulation of activated CD8+ T-cells with rhIL-23 served as a positive control. IL-17 production by activated CD8+ T-cells was dose dependently upregulated by rhIL-23. Similarly, the addition of supernatants of TLR2 activated monocytes to CD8+ T-cell cultures resulted in an 1.4±0.2 fold upregulation of IL-17 and supernatants of TLR4 activated monocytes in a 3.6±0.2 fold

upregulation of IL-17 production by activated CD8+ T-cells. In contrast, treatment of CD8+ T-cells with supernatants of TLR2, 3 or 4 activated RASF had no effect on the IL-17 production. These results confirmed that RASF stimulated with TLR ligands, although expressing the p19 subunit, do not produce bioactive IL-23 in contrast to TLR activated monocytes.

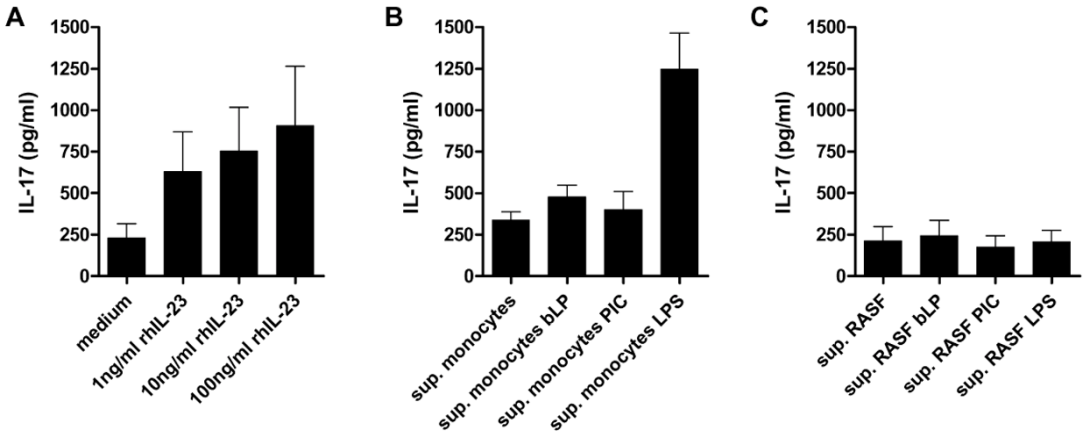

Figure 6: Monocytes but not RASF secrete bioactive IL-23 after TLR activation. CD8+ cells were preactivated with anti-CD3 and anti CD-28 antibodies and stimulated with rhIL-23 (A), supernatants of TLR2 ,3 and 4 activated monocytes (B) or supernatants of TLR 2, 3 and 4 activated RASF (C). IL-23 dependent induction of IL-17 protein by CD8+ cells was determined by ELISA.

Discussion

In the current study we demonstrate that p19 is expressed and induced independently of p40 in RASF in contrast to MDDCs and monocytes. In addition we show that heterodimeric IL-23 is present at unexpectedly low levels in joints of RA patients.

Recent reports have indicated the involvement of IL-23 in several autoimmune diseases including colitis, psoriasis and arthritis. Treatment with anti-p19 antibodies in mice inhibited central nervous system autoimmune inflammation, prevented active colitis and exacerbated lyme arthritis [19-21]. In the collagen induced arthritis mouse model, p19 and p40 are essential for the development of joint inflammation [9]. However, studies regarding the role of IL-23 in RA have not been published so far. We show that IL-23 is present at low levels in joints of RA but barely detectable in OA. Only a small number of p19/p40 double positive cells were detected in RA synovial tissues. In accordance with this finding we could detect heterodimeric IL-23 in only 5 out of 28 patients. This result was unexpected as high p40 and

high p19 levels were described in earlier reports [7, 10]. Interestingly, p40 seemed to be the limiting factor for the production of IL-23 as most synoviocytes were single positive for p19. In addition we found p40 positive/p19 negative cells. We presume that these cells secrete the related cytokine IL-12, which is composed of the p40 and p35 subunits. In accordance IL-12 positive cells have been described in the sublining layer of the RA synovium [22].

The role of IL-23 in autoimmune diseases is suggested to be dependent on the promotion and proliferation of IL-17-producing Th17 T-cells [23]. The Th17 subset was recently described as a third subset of T helper cells in addition to the well known Th1 and Th2 subsets. Th17 T-cells were shown to be associated with autoimmune diseases. For example in a mouse model of experimental colitis, IL-23 induced disease by stimulation of Th17 T-cells [24]. IL-23 signals through a heterodimeric receptor composed of IL-12Rβ1 and IL-23R. Whereas IL12β1 is expressed constitutively, IL-23R is not expressed on naive T cells. IL-6 and TGF-beta induce the expression of IL-23R and are essential for the differentiation of naïve T cells into Th17 cells. Consequently, this subset becomes responsive for IL-23, which is crucial for the expansion and survival of Th17 cells [5]. In analogy the elevated levels of IL-6 and TGF-beta in the joints of patients with RA might differentiate naive T cells into Th-17 cells, whereas IL-23 might be involved in the persistence of inflammation.

IL-23 was shown to be expressed by activated dendritic cells, monocytes, keratinocytes and microglia [18, 25-27]. The activity of inflammatory cells can be influenced by the activation of TLRs that interact with microbial or endogenous ligands. In earlier reports we have demonstrated that TLR2, 3 and 4 expression is elevated in RA synovial tissues [28-31]. Endogenous TLR ligands, such as heat shock proteins and necrotic cells, are present in the chronically inflamed joints and may lead to a sustained activation of TLR signalling pathways in RA. In the current study we show differential expression and induction of the IL-23 subunits by TLR2, 3 or 4 ligands in RASF, monocytes and MDDC. We found that TLR activation induced p19 but not p40 protein expression in RASF whereas in MDDCs and monocytes both IL-23 subunits were upregulated. Stimulation of pre-activated CD8+ T-cells with rhIL-23 as well as with supernatants of TLR activated monocytes but not TLR activated RASF led to an increase in the production of IL-17. Therefore, we assume that activated dendritic cells and monocytes or macrophages are the main producers of heterodimeric IL-23 in the RA synovium whereas RASF express p19 independently of p40. This is the first study showing differential expression of IL-23 subunits in the synovium. Consequently p19

expression does not necessarily correlate with the presence of bioactive IL-23 as has been assumed in previous reports [10, 32].

Ubiquitous transgenic expression of p19 in mice results in a phenotype of systemic inflammation, impaired growth and premature death [33]. Additionally, high levels of TNF-α and IL-1β were detected in the circulation of p19 transgenic mice. It has been suggested that the development of multiorgan inflammation in p19 transgenic mice is dependent on the dimerization of p19 with p40 and therefore dependent on the heterodimer IL-23. However, the p40 subunit was not detectable in serum of p19 transgenic mice. These findings together with our data showing a large number of p19 positive / p40 negative synoviocytes in the RA synovial lining layer and at sites of invasion, gives further evidence that p19 may be implicated in the RA pathogenesis independently of its dimerization with p40. The p19 protein shares homology with members of the IL-6/IL-12 family which includes IL-6, oncostatin M, G-CSF and p35. It has been speculated that additional heterodimeric complexes might exist in the IL-12 family which comprises IL-12, IL-23 as well as IL-27. For example combinations of Epstein-Barr virus-induced gene 3 (EBI3) and p35 as well as EBI3 and p19 have been described [34]. Whether p19 might have additional binding partners other than p40 in vivo needs further investigation.

In conclusion we report abundant expression of p19 but not heterodimeric IL-23 by RASF at sites of invasion and in vitro upon stimulation by TLR ligands. The differential expression of p19 and p40 suggests that p19 does not necessarily indicate the presence of active IL-23 and gives evidence for a p40 independent involvement of p19 in the pathogenesis of RA

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CHAPTER 6 Appendix

Abbreviations

AP	alkaline phosphatase
AP-1	activator protein-1
APC	antigen presenting cell
CC	chemokine
CCL	chemokine ligand
cDNA	complementary DNA
CpG	deoxy-cytidylate-phosphate-deoxy-guanylate
DC	dendritic cell
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotriphosphate
ECM	extracellular matrix
FADD	Fas-associated via death domain
FAM	carboxyfluorescein
FCS	fetal bovine serum
HLA	human histocompatibility leukocyte antigen
HRP	horseradish peroxidase
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IFN	interferone
Ig	immunoglobuline
IkB	inhibitor of kappa light chain gene enhancer in B cells
IkK	IkB kinase
IL	interleukin
IRAK	interleukin-1 receptor- associated kinase
IRF	interferon regulatory factor
JNK	Jun N- (amino-) terminal kinase
Mal	MyD88-adaptor like; = TIRAP
MAPK	mitogen-activated kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88
NBT	nitro blue tetrazolium
NF-kB	nuclear factor kappa B
OA	osteoarthritis
ODN	oligodeoxynucleotide
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBEF	pre-B cell colony-enhancing factor
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RASF	RA synovial fibroblasts
RF	rheumatoid factor
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription PCR
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TAMRA	tetramethy rhodamine
TIMP	tissue inhibitor of metalloproteinase
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor

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Brentano F*, Ospelt C*, Rengel Y, Stanczyk J, Kolling C, Tak PP, Gay RE,Gay S, Kyburz D. Overexpression of toll-like receptor 3 and 4 in early rheumatoid arthritis synovial tissue. (*contributed equally). Arthritis Rheum. *In revision.*

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Differential induction of IL-23 subunits by TLR ligands in rheumatoid arthritis synovial fibroblasts and monocytes.

Japanese College of Rheumatology (JCR) 2007, Yokohama, oral presentation.
Proinflammatory and matrix degrading activity of PBEF/Visfatin in the pathogenesis of rheumatoid arthritis.

American College of Rheumatology (ACR) 2006 , Washington, oral presentation.
Increased expression and proinflammatory activity of PBEF / Visfatin in rheumatoid arthritis.

Center of Medical Research, Zurich, March 2006, poster presentation
Induction of PBEF in rheumatoid arthritis synovial fibroblasts stimulated with TLR ligands. (awarded for best poster).

American College of Rheumatology (ACR) 2005 , San Diego, poster presentation.
RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via TLR3

5th Global Arthritis Research Network (GARN) 2005, Vienna, oral presentation.
TLR3 dependent activation of rheumatoid arthritis synovial fibroblasts by RNA of synthetic and endogenous origin.

American College of Rheumatology (ACR) 2004 , San Antonio, poster presentation.
TLR expression in synovial fibroblasts and normal skin fibroblasts and induction of matrix metalloproteinases expression by various TLR ligands.

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